Developmental Biology **252,** 151–169 (2002)
doi:10.1006/dbio.2002.0861

REVIEW

Early Steps in the Formation of Neural Tissue in Ascidian Embryos

P. Lemaire,[1] V. Bertrand, and C. Hudson[2]

Institut de Biologie du Développement de Marseille, Laboratoire de Génétique et Physiologie du Développement, CNRS Université de la Méditerranée, Case 907, Campus de Luminy, F-13288 Marseille, France

Ascidians are simple invertebrate chordates whose lineage diverged from that of vertebrates at the base of the chordate tree. Their larvae display a typical chordate body plan, but are composed of a remarkably small number of cells. Ascidians develop with an invariant cell lineage, and their embryos can be easily experimentally manipulated during the cleavage stages. Their larval nervous system is organised in a similar way as in vertebrates but is composed of less than 130 neurones and around 230 glial cells. This remarkable simplicity offers an opportunity to understand, at the cellular and molecular levels, the ontogeny and function of each neural cell. Here, we first review the organisation of the ascidian nervous system and its lineage. We then focus on the current understanding of the processes of neural specification and patterning before and during gastrulation. We discuss these advances in the context of what is currently known in vertebrates.

Key Words: **ascidians; chordates; evolution; nervous system; neural induction; embryo; development; neurone; Otx; BMP; FGF; proteases; CNS; PNS; placodes; neural crest.**

INTRODUCTION

Understanding the ontogenesis and function of the nervous system constitutes one of the most fascinating goals of modern biology. During the past decade, important progress has been made in our understanding of the formation of the vertebrate nervous system. To greatly simplify, several steps can be distinguished in this process (Sasai, 1998). Cells are first induced to adopt a neural identity before and during the first part of gastrulation. The neural plate is then regionalised along the anteroposterior and mediolateral axes during gastrulation. Finally, differentiation of specific cell types ensues.

Progress in our understanding of the first of these steps, the induction of neural tissue, has led to apparently conflicting models in lower vertebrates and in amniotes. In lower vertebrates such as *Xenopus*, the central nervous system originates from the animal dorsal territories. A popular model proposes that neural fate is the default state, obtained by preventing epidermis induction by factors of the bone morphogenetic protein (BMP) family (Harland, 2000). Consistent with this model, *Bmp* genes are repressed in the animal dorsal territories, while inhibitors of BMPs are secreted from the equatorial dorsal territories (the organiser). Overexpression of *Bmp2/4* prevents neural induction and promotes epidermal differentiation, whereas ectopic expression of BMP inhibitors, such as Chordin and Noggin, promotes neural differentiation at the expense of epidermis. The model proposed in amniotes differs from the *Xenopus* model in that inhibition of BMP signalling is required, but not sufficient, to promote neural differentiation (Wilson and Edlund, 2001). In contrast, a major role has been proposed for FGF during the earliest steps of neural induction, a role which is not generally accepted in *Xenopus* (Wilson and Edlund, 2001). While these apparent discrepancies may finally be resolved and a unified mechanism proposed, they illustrate the fact that, in spite of a common body plan, different early vertebrate embryos often appear to use distinct developmental strategies. This diversity makes it difficult to identify the ancestral strategies that led to the emergence of the vertebrate nervous system.

Studying invertebrate chordates (Tunicates and Cephalochordates; Fig. 1A) may help solve this issue, as any conserved strategy between these animals and at least some

[1] To whom correspondence should be addressed. Fax: +33-4-91820682. E-mail: lemaire@lgpd.univ-mrs.fr.

[2] Current address: Unité de Biologie du Développement (UMR 7009), CNRS/UPMC, Station Zoologique, Observatoire Océonologique, Villefranche-Sur-Mer, F-06230, France.

0012-1606/02 $35.00

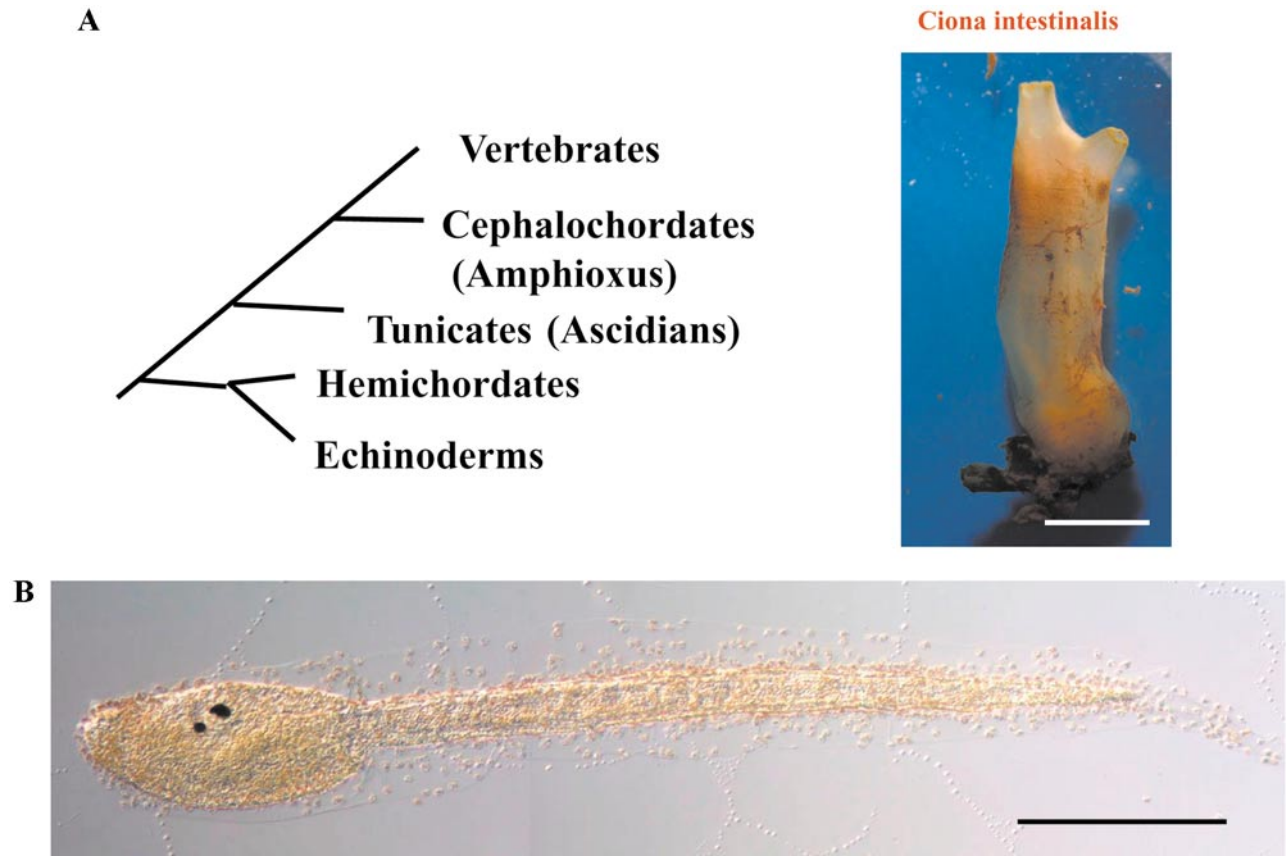

FIG. 1. Ascidians are invertebrate chordates. (A) Phylogenetic tree of the deuterostomes. A side view of an adult ascidian (*Ciona intestinalis*) is shown at the right. Scale bar, 1 cm. (B) Lateral view of a *C. intestinalis* tadpole. Note the long tail and characteristic pigment cells in the trunk/head. Scale bar, 100 μm

vertebrates is likely to have been used by the last common ancestor of these phyla. Invertebrate chordates and vertebrates share a common larval body plan consisting of a central notochord flanked by muscle blocks, a dorsal hollow neural tube, and ventral endoderm. Tunicate and Cephalochordate embryos, however, are much simpler than those of vertebrates both in terms of anatomy and of cell numbers. The Cephalochordates (*Amphioxus*) are the closest relatives of the vertebrates. The study of their nervous system has shown that its organisation is similar to that of vertebrates, except that structures such as the neural crest and the telencephalon are likely vertebrate inventions (Holland and Holland, 1999). In addition, analysis of the expression of signalling molecules such as BMP or SHH suggests that at least some of the mechanisms used to regionalise or specify the neural tissue have been conserved between vertebrates and *Amphioxus*. *Amphioxus* embryos are however very difficult to experimentally manipulate, and thus no functional data can be collected in support of a conserved role for these signalling pathways.

Ascidians diverged from the vertebrate lineage earlier than Cephalochordates (Fig. 1A). The fossil record provides evidence that they already existed during the lower Cambrian (Shu *et al.*, 2001). While their adult body plan is at odds with vertebrates, their very simple larvae exhibit a typical chordate body plan (Fig. 1B). The application of modern molecular biology techniques to their embryos has recently spurned considerable interest. These studies complemented work in *Amphioxus* by providing functional data on the mechanisms of neural specification in an invertebrate chordate. In this review, we will first introduce ascidians and their larval nervous system, before reviewing recent progress on our understanding of neural specification and patterning before and during gastrulation.

THE ASCIDIAN SYSTEM

Ascidians are marine organisms that initially came to fame at the end of the 19th century and beginning of the 20th century. During most of the 19th century, scientists attempted to understand embryos by simply observing and describing their normal development. In 1887, using ascidian embryos, L. Chabry succeeded for the first time in

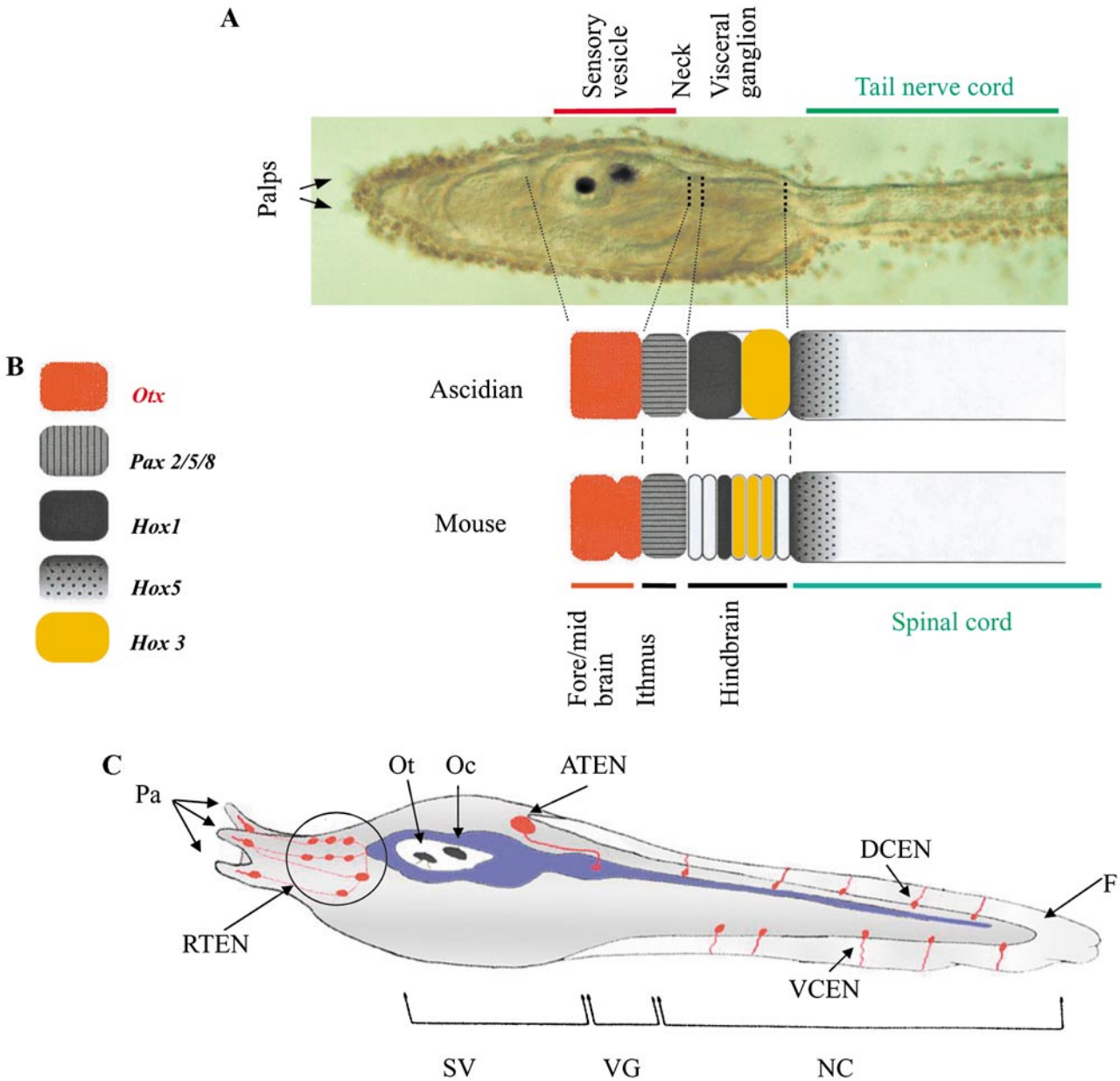

FIG. 2. Organisation of the ascidian nervous system. (A) Lateral view of the anterior section of a *C. intestinalis* larva, revealing the coarse organisation of the nervous system. (B) Schematic representation of the expression pattern of conserved genes between ascidians and vertebrates. The name of the vertebrate territories thought to be homologous to the sensory vesicle, neck, visceral ganglion, and tail nerve cord is indicated. Adapted from (Wada *et al.*, 1998). (C) Position of the epidermal sensory neurones of the PNS (red). The CNS is represented in blue. Pa, palps; RTEN, rostral trunk epidermal neurones; Ot, otolith; Oc, ocellus; ATEN, apical trunk epidermal neurones; VCEN, ventral caudal epidermal neurones; DCEN, dorsal caudal epidermal neurones; F, Fin. The position of the axones, when known, is indicated. Adapted from Nicol and Meinertzhagen (1991).

interpreting the effect of experimental manipulations on embryonic development (Chabry, 1887), and thus became one of the founders of modern experimental embryology. A few decades later, E. G. Conklin (Conklin, 1905) pioneered cell lineage studies by describing the lineage of ascidian embryos. In this study, he identified a specialised cytoplasmic domain in fertilised ascidian eggs, the myoplasm, which is preferentially inherited by embryonic muscle cells. These experiments led to the idea that inheritance of a localised maternal determinant could be sufficient to

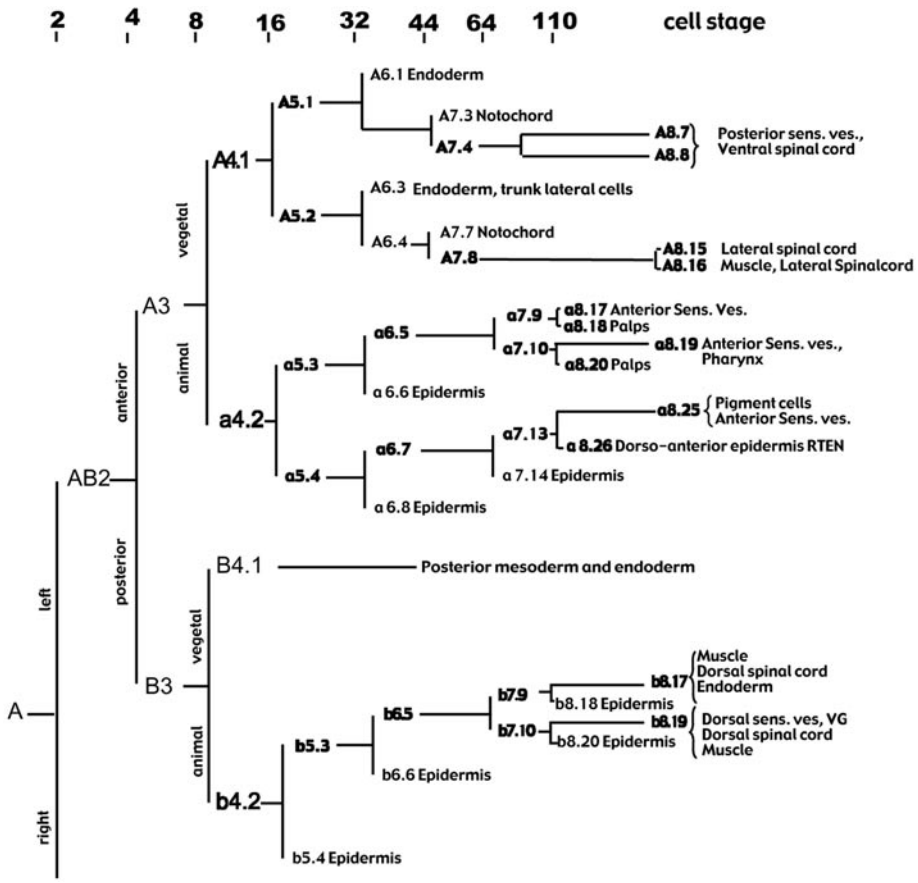

FIG. 3. The neural lineages. The tree only represents the left lineages. The right lineages are identical. The blastomeres that contribute to the nervous system are in bold. The nomenclature of the cells is as follows: The letter indicates the origin of the blastomere at the 8-cell stage (A, anterior vegetal; B, posterior vegetal; a, anterior animal; b, posterior animal). The first number indicates the number of the cleavage + 1. For example A4.1 is a 3rd cleavage anterior vegetal blastomere. The second number indicates the identity of the blastomere in a given lineage at a given stage. This number increases with the distance from the vegetal pole. It doubles at each generation (e.g., A4.1 gives rise to A5.1 and A5.2; A5.2 gives rise to A6.3 and A6.4, etc.).

determine the fate of a cell (Gilbert, 1997; Pourquie, 2001). This mode of development was called mosaic development and is used in the embryos of many phyla.

In spite of their key phylogenetic position and contribution to the birth of modern embryology, ascidians, like most marine organisms, have slowly moved to the backstage in the course of the 20th century. Yet, starting from the early 1980s, William Jeffery and Noriyuki Satoh introduced molecular biology to ascidian studies and greatly contributed to the present renewal of interest in the developmental biology of these animals (Corbo *et al.,* 2001). The past 5 years have seen the application to ascidian embryology of an impressive number of molecular tools. These include transgenesis by electroporation of eggs with plasmid DNA (Corbo *et al.,* 1997b), loss-of-function studies using antisense morpholino oligonucleotides (Satou *et al.,* 2001a), and the near completion of the sequencing of the genomes of two closely related species, *Ciona intestinalis* and *Ciona savignyi* (URLs: http://www.jgi.doe.gov/programs/Ciona/Ciona_mainpage.html, http://ghost.zool.kyoto-u.ac.jp/, and http://www-genome.wi.mit.edu/annotation/Ciona/). Because ascidians radiated from the vertebrate branch before the two rounds of vertebrate genome duplications (Simmen *et al.,* 1998), their genomes are remarkably small and simple, harbouring, for example, a single Hox cluster (Di Gregorio *et al.,* 1995). The combination of the ease of promoter analysis by electroporation, the availability of sequences from two closely related species, and small genome size make *Ciona* a powerful system to study gene circuitries underlying developmental processes. In addition, *Ciona* can now be bred in the lab and was shown to have a life cycle of only 10 weeks, shorter than most vertebrates. The simple genome, short life cycle, and hermaphroditic nature of ascidians have encouraged several groups to

embark upon a genetic analysis of ascidian development (Moody *et al.,* 1999; Nakatani *et al.,* 1999; Sordino *et al.,* 2001).

The small cell number, invariant lineage, and ease of performing embryological experiments on ascidian embryos also justifies their current popularity. Vertebrate embryos develop with a large number of cells: for example, 20,000 cells in a young *Xenopus* gastrula, compared with 110 cells in an ascidian embryo at the same stage. Fully formed *Ciona* larvae contain around 2600 cells. In addition, unlike in vertebrates, the cleavage patterns are invariant between embryos, which makes it possible to unambiguously identify, name, and determine the fate of each cell in an early gastrula (Nishida, 1987). Finally, some ascidian species offer optically transparent embryos, which can be used to image cellular behaviours and morphogenesis in living embryos (Munro and Odell, 2002).

Taken together, these characteristics make ascidians a favourable model system to obtain a detailed understanding of the successive molecular and cellular processes that dictate the fate of each embryonic cell.

ORGANISATION AND EVOLUTIONARY CONSERVATION OF THE ASCIDIAN NERVOUS SYSTEM

The ascidian central nervous system (CNS) consists of less than 100 neurones and 250 glial cells (Meinertzhagen and Okamura, 2001). The peripheral nervous system (PNS) is composed of 20–30 epidermal sensory neurones, which have been precisely mapped in developing larvae (Ohtsuka *et al.,* 2001b; Takamura, 1998).

The CNS

As in vertebrates, the ascidian CNS develops from a neural plate, which is regionalised along its anteroposterior (AP) and dorsoventral (DV) axes. The anteriormost part of the neural plate will not contribute to the CNS and does not roll up. It forms the dorsoanterior epidermis, which includes the adhesive organ (or palps), head sensory neurones, and the pharynx. The 3 palps form a docking structure for the fixation of the larva on the substrate during metamorphosis. The pharynx gives rise to the branchial siphon of the postmetamorphic juvenile. The rest of the neural plate rolls up into the dorsally located CNS, which consists of the sensory vesicle, the neck, the visceral ganglion, and the tail nerve cord (Fig. 2A). The sensory vesicle contains the neurohypophysis and three sensory organs. These are the otolith, which is thought to function in geotactic responses; the light-sensing ocellus; and the so-called pressure organ, which is thought to be involved in either hydrostatic pressure measurement, chemosensation, or secretion (Nicol and Meinertzhagen, 1991). The sensory vesicle is also the site of the birth of most of the 80–100 CNS neurones. In *Ciona,* the part of the sensory vesicle which is located posterior to the ocellus probably corresponds to what has been designated as the anterior part of the brain stem in *Halocynthia* (Nishida, 1987). Because of the imprecision of the term brain stem in vertebrates, we prefer to refer to this territory as the posterior sensory vesicle in agreement with several *Ciona* authors (Meinertzhagen and Okamura, 2001; Nicol and Meinertzhagen, 1991; Takamura, 1998). While the function of the neck (6 cells in *Ciona*) is unclear, the visceral ganglion of *Ciona* (it is not morphologically recognisable in *Halocynthia roretzi*) contains 45 cells, including 18 neurones, of which 5 pairs are motoneurones (Nicol and Meinertzhagen, 1991). *Halocynthia* larvae have 3 pairs of motoneurones in a similar position (Meinertzhagen and Okamura, 2001; Okada *et al.,* 2002). Finally, the tail nerve cord constitutes a simple neural tube that extends along the larval tail and is thought to contain exclusively ciliated ependymal cells. The absence of neurones in the tail nerve cord is probably a derived character, which may not represent the primitive chordate condition (Wada and Satoh, 2001).

The structures of the ascidian CNS can be likened to vertebrate structures on the basis of morphology and gene expression studies (Fig. 2B). However, comparative analysis is a difficult exercise due to the current poor anatomical description of the ascidian nervous system and the small number of marker genes yet available. Therefore, the following summary of the current evidence for homologies between the ascidian and vertebrate CNS should be taken with care.

The sensory vesicle expresses *Otx* and is therefore thought to correspond to the vertebrate prosencephalon and mesencephalon. Interestingly, the ascidian orthologue of the vertebrate telencephalon marker *Emx* is expressed in the dorsoanterior epidermis but not in the sensory vesicle (Oda and Saiga, 2001). One interpretation of this finding may be that ascidians lack a telencephalon. A similar proposition has been made for *Amphioxus* (Holland and Holland, 2001). At the most anterior aspect of the sensory vesicle, is an infundibulum-like structure connected to the pharynx, which has been proposed to be homologous to the vertebrate neurohypophysis (Manni *et al.,* 1999). The otolith and ocellus both contain melanised pigment cells. The position of these cells is consistent with a prosencephalic origin. This and the expression of *tyrosinase* (Sato *et al.,* 1997), *Otx* (Hinman and Degnan, 2000; Hudson and Lemaire, 2001; Wada *et al.,* 1996), *Msxb* (Aniello *et al.,* 1999), and *Pax6* (Glardon *et al.,* 1997) in pigment cell precursors, suggest that these cells are homologous to retinal pigment epithelial (RPE) cells of the eye or pineal gland (Sato and Yamamoto, 2001). The embryological origin of these cells in the lateral neural plate and their expression of *Pax3/7* (Wada *et al.,* 1997) support a homology with pineal cells. This will need confirmation using a larger set of markers.

The neck is found immediately posterior to the *Otx* expression domain. It expresses *Pax2/5/8* but not *Hox1.* This structure may thus represent the ascidian equivalent of the vertebrate midhindbrain boundary (MHB) (Wada *et*

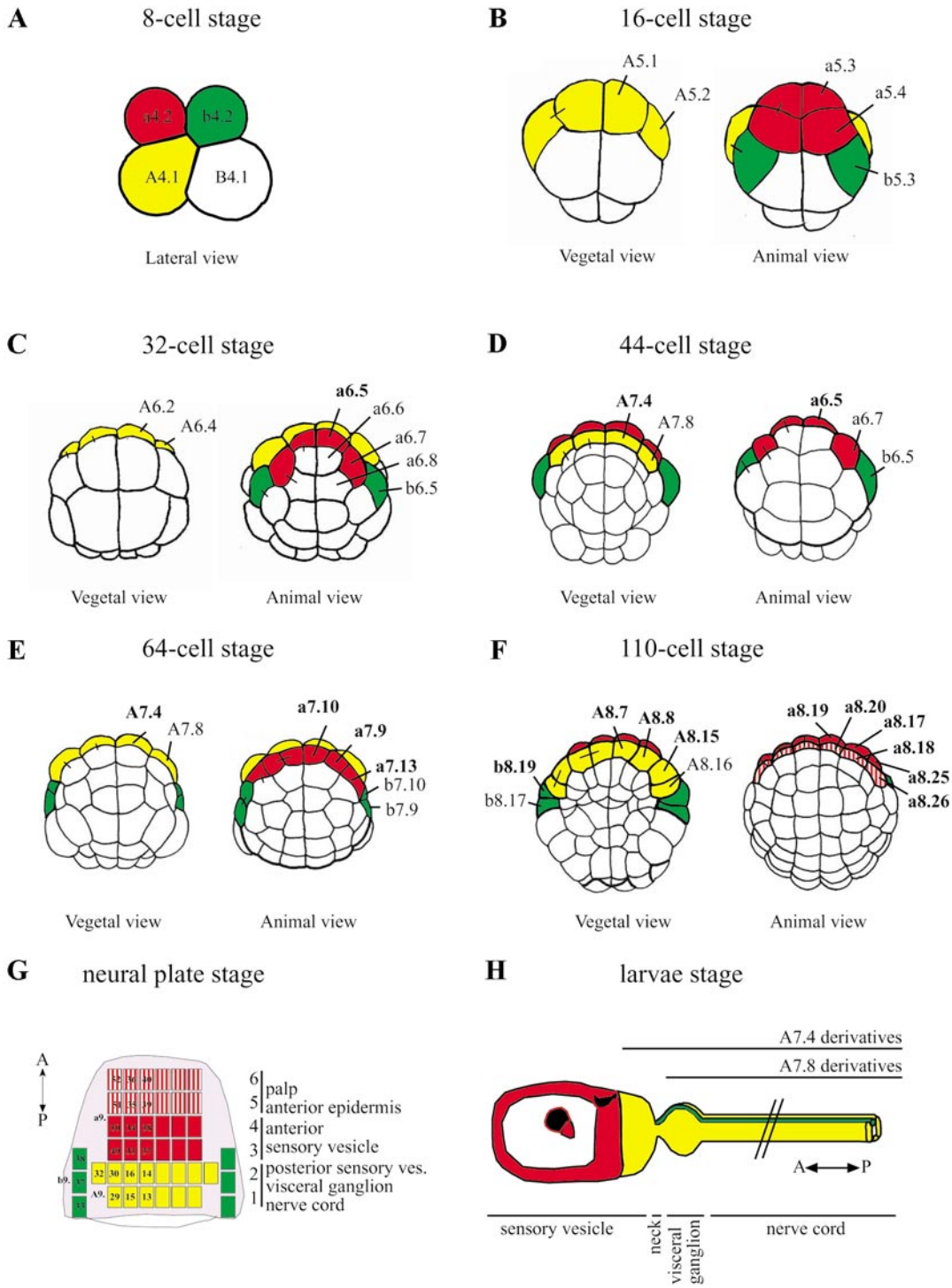

FIG. 4. Position of the neural precursors in developing embryos. Blastomeres coloured red denote those which derive from a4.2 at the 8-cell stage, yellow-coloured blastomeres denote those which derive from A4.1, and green blastomeres those from b4.2. Only those blastomeres which contain neural fates contain these colours; other cells which also belong to these lineages but do not contain neural fates are left uncoloured. For those blastomeres containing neural fates, bars mark blastomere sisters. Blastomeres which are fate restricted to give rise only to neural plate structures have their blastomere names in bold. Dorsal–anterior epidermis and sensory vesicle fates separate at the 110-cell stage. The dorsal–anterior epidermal blastomeres are henceforth hashed in red. Both animal and vegetal views are shown for each stage, except (A) 8-cell stage, in which a lateral view is shown. (G) Flat projection of neural plate at neural plate stage. Anterior up, dorsal view. (H) Schematic view of larval central nervous system showing sensory vesicle (containing pigment cells) neck, visceral ganglion, and tail nerve cord. The tail nerve cord is greatly reduced in length on the scheme and a cross-section (far right) shows it to be only four cells in cross-section. Peripheral neural structures are not indicated. Anterior left, lateral view.

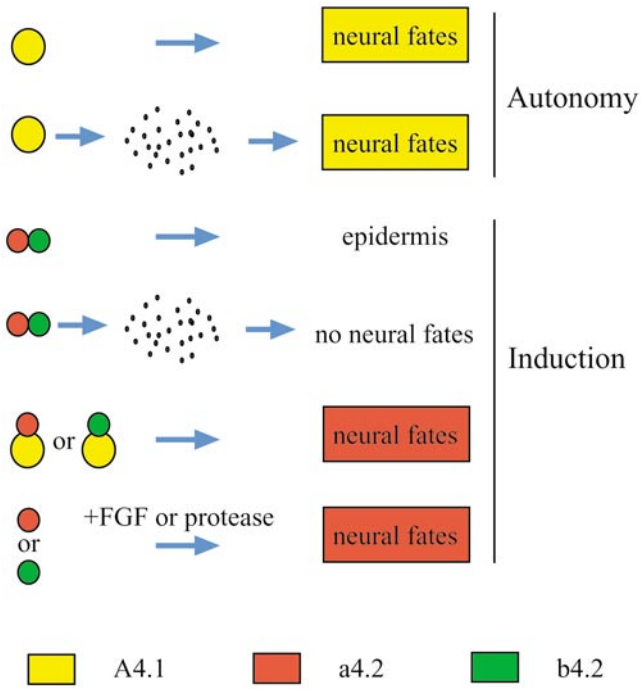

FIG. 5. Two different modes of neural specification. Ascidians contain both neural tissue which is formed autonomously and that which is induced. Autonomous: When A4.1 blastomeres are cultured in isolation, some of the resulting blastomeres will adopt a neural fate. Even if this blastomere is cultured in continuous dispersion, some of its derivatives will adopt a neural fate, suggesting that this is a cell-autonomous process. Induced: a4.2 and b4.2 blastomeres cultured in isolation will form epidermis. The same occurs if they are cultured in continuous cell dispersion. However, when cultured in contact with A4.1 blastomere, or in the presence of protease or bFGF, some of the animal cells will adopt a neural fate.

al., 1998). There is as yet, however, no evidence that the neck has organiser properties comparable to its vertebrate counterpart. It will be interesting to test whether genes for classical MHB markers, such as Engrailed, or for secreted organising factors, such as FGF8 or Wnt1, are also expressed in the neck.

The visceral ganglion expresses *Hox1* anteriorly (Katsuyama *et al.*, 1995) and *Hox3* posteriorly (Locascio *et al.*, 1999). In spite of its lack of visible segmentation, this structure may therefore be homologous to the vertebrate caudal rhombencephalon (rhombomeres 4–6). However, in contrast to vertebrates, no obvious segmentation is visible in the visceral ganglion and it will be interesting to determine the expression of the orthologues of vertebrate rhombomere-specific genes, such as Krox-20, Eph receptors, or Kreisler (Schneider-Maunoury *et al.*, 1998). Finally, the anterior tail nerve cord expresses *Hox5*, which is expressed in the vertebrate anterior spinal cord (Gionti *et al.*, 1998).

Regionalisation of ascidian and vertebrate neural tubes along the dorsoventral (D-V) axis is also comparable. As in vertebrates, *HNF3β* is expressed in the ventralmost cells of the neural tube, which may be akin to the floorplate (Corbo *et al.*, 1997a; Shimauchi *et al.*, 1997). Expression of *Gsx*, like vertebrate *Gsh*, is detected in intermediate cells of the neural tube, though expression in ascidians is restricted to the anterior nervous system (Hudson and Lemaire, 2001). Expression of ascidian *Snail*, *Pax3/7*, *Bmpb*, *Msxb*, and *distal-less* (*Dll1*) is detected during gastrulation in the lateral cells of the neural plate or in the flanking future dorsal epidermis (Aniello *et al.*, 1999; Corbo *et al.*, 1997a; Wada *et al.*, 1997, 1999; Wada and Saiga, 1999a). This parallels the expression in the dorsal most territories of the neural tube or adjacent epidermis of the vertebrate orthologues of these genes (Mayor and Aybar, 2001).

Taken together, the available data on the AP and DV regionalisation of the ascidian central nervous system suggest that its structure, though much simpler, has overall homology to that of vertebrates and may be patterned by similar mechanisms as the vertebrate CNS.

The PNS

The PNS consists of epidermal sensory neurones (ESNs) found in the head and tail (Fig. 2C). These neurones extend long cilia into the tunic of the ascidian larvae and have therefore been proposed to constitute mechanosensory organs (Crowther and Whittaker, 1992; Torrence and Cloney, 1982). Their position in larvae has been described in several ascidian species (Ohtsuka *et al.*, 2001b; Takamura, 1998; Torrence and Cloney, 1982; Yagi and Makabe, 2001). The number of caudal epidermal sensory neurones varies within individuals of one species. They are found at more or less regular intervals in the epidermis of the dorsal and ventral midlines. Larval trunk epidermal sensory neurones are more precisely positioned and show less variability between individuals. Two sensory neurones are found in each of the 3 palps, or papillae, at the anterior tip of the larvae. In *Ciona*, the palp ESNs are connected to the sensory vesicle via connections with around 10 precisely positioned rostral trunk epidermal neurones (RTEN)(Takamura, 1998). In addition, 2 groups of 2, or sometimes more, apical trunk epidermal neurones (ATEN) are found dorsal to the posterior part of the sensory vesicle and make connections with the posterior part of the visceral ganglion (Takamura, 1998). The function of these neurones is unknown. The arrangement of the trunk ESNs in *Halocynthia* larvae, marked by the expression of the actin-binding protein gelsolin, is very similar (Ohtsuka *et al.*, 2001b).

In vertebrates, the PNS is derived from the neural crest and the placodes (Holland and Holland, 2001). There are no morphologically recognisable, delaminating, neural crest cells in invertebrate chordates (Holland and Holland, 2001). However, the expression of *Pax3/7*, *Distal-less*, *Msx*, *Bmp2/4*, and *Snail* in the dorsal neural tube of ascidians and vertebrates is conserved. Furthermore, the ascidian PNS appears to be derived from this population of cells. Some of

the *Halocynthia* head ESNs, probably the RTENs, originate at the 110-cell stage from a blastomere located in the lateralmost row of the neural plate and which transiently expresses *Pax3/7* (Ohtsuka *et al.*, 2001b; Wada *et al.*, 1997; Yagi and Makabe, 2001). Similarly, the tail ESNs originate from dorsal tail epidermal blastomeres (Ohtsuka *et al.*, 2001b). Judging from the lineage description (Nishida, 1987), these cells also transiently express Pax3/7 (Wada *et al.*, 1997). Thus, cells which express a neural crest-like genetic programme generate cell types similar to vertebrate neural crest-derived cells. This suggests that a specific population of cells displaying several neural crest properties, but lacking migratory behaviour, already existed in invertebrate chordates. Placodes, like the neural crest, were also thought to have emerged with the vertebrates. Two ascidian structures, however, have been likened to placodes: the pharynx and the atrial siphon primordia. These structures are formed by an epidermal thickening, followed by invagination (Katz, 1983). They form sensory organs, which, after metamorphosis, contain ciliated sensory cells resembling those of the vertebrate inner ear (Bone and Ryan, 1978). This anatomical similarity and the expression of *Pax2/5/8* in these primordia have led to the proposition that the atrial primordia are true placodes, homologous to the vertebrate inner ear (Wada *et al.*, 1998). Analysis of further orthologues of vertebrate placode genes, such as Six1 (Pandur and Moody, 2000), will be needed to validate this proposition.

EMBRYOLOGICAL ORIGIN OF THE NERVOUS SYSTEM: DESCRIPTION OF THE NEURAL LINEAGES

Ascidian neural tissue derives from three lineages: the anterior vegetal A-line and the animal a- and b-lines (Fig. 3) (Nicol and Meinertzhagen, 1988a,b; Nishida, 1987). The existence of an invariant lineage enabled the origin of the different territories of the nervous system to be traced up to the midgastrula stage (Fig. 4). At this stage, the neural plate is a remarkably geometric structure consisting of a chessboard of 44 cells (Fig. 4G). The lineages described below are thought to be identical or very similar in the two most studied ascidians, *Halocynthia* and *Ciona*. A summary of the rules that guide the naming of blastomeres is found in the legend to Fig. 3.

A-Line Cells

At larval stages, A-line neural cells form the lateral and ventral cells of the posterior sensory vesicle, neck region, visceral ganglion, and tail nerve cord (depicted in yellow in Fig. 4H). These cells originate at the 8-cell stage from the A4.1 blastomere, which also contains other fates, such as endoderm, notochord, and trunk lateral cells (Nishida and Satoh, 1989; Nishida, 1987). The lineage of A-line neural cells is shown in Fig. 3. One pair of A-line cells, A7.4, becomes fate restricted to a neural fate at the 44-cell stage (Figs. 3 and 4). This blastomere gives rise to the ventral tail nerve cord as well as the ventral and lateral posterior sensory vesicle, neck, and visceral ganglion. The neighbouring cell, A7.8, gives rise to the lateral visceral ganglion and tail nerve cord, but is not yet fate restricted. At the 110-cell stage, A7.8 cleaves to produce A8.15, which is restricted to a neural fate, and A8.16, which is still bipotential. During the next cell division, A8.16 cleaves to generate blastomeres restricted to muscle (A9.31) and neural (A9.32) fates. By this stage, A7.4 has generated 8 A-line neural precursors and A7.8 has generated 6. These cells occupy the 2 posteriormost rows of the neural plate. The position of each of these blastomeres in the embryo can be seen in Fig. 4.

a-Line Cells

The a-line neural plate cells form the anterior sensory vesicle, including the characteristic sensory pigment cells, as well as to the dorsal–anterior epidermis, including palps and head epidermal sensory neurones (Nishida, 1987). The lineage of a-line cells can be seen in Fig. 3. Epidermal and neural plate fates begin to segregate at the 32-cell stage. The a6.5 blastomere is restricted to anterior neural plate and the a6.6 and a6.8 blastomeres are restricted to epidermis. The other a-line neural plate cells come from the a6.7 blastomere of the 32-cell stage, the progeny of which becomes fate restricted shortly after, at the 64-cell stage. Hence, at the 64-cell stage, 3 neural plate restricted blastomere pairs constitute the entire anterior neural plate precursors. These are a7.10 and a7.9 (daughters of a6.5) and a7.13 (a daughter of a6.7). At the 110-cell stage, these cells all cleave along the AP axis to give (1) anterior a-line neural plate precursors (a8.20, a8.18, a8.26), which give rise to the dorsal-anterior epidermis, and (2) posterior a-line neural plate precursors (a8.19, a8.17, a8.25), which give rise to the anterior sensory vesicle. The a6.5 precursors give rise to the ventral and lateral anterior sensory vesicle (a8.17/19), as well as the palps (a8.18/20), the a6.7 precursors to the dorsal anterior sensory vesicle (a8.25), and dorsoanterior epidermis (a8.26). Of these latter cells, the pigment cells come from the a8.25 blastomere and the head epidermal sensory neurones from a8.26. The position of each of these cells in the embryo can be seen in Fig. 4.

b-Line Cells

Most b-line cells form epidermis. Only a few daughter cells of b4.2 will be part of the neural plate. These cells contribute to the dorsal most cell of the neural tube. They become fate restricted relatively late in development (Nishida, 1987). The b-line lineage can be seen in Fig. 3 and the positions of the precursors on Fig. 4. One b-line neural cell, b8.19, becomes fate restricted at the 110-cell stage. At this stage, another b-line blastomere, b8.17, also contains neural fate but it is not fate restricted. At the neural plate stage, this blastomere cleaves to generate the b9.33 blastomere, which contributes to the dorsal neural tube.

DISTINCT MECHANISMS SPECIFY THE NEURAL FATE IN A-, a-, AND b-LINE CELLS

Embryological evidence has shown that the different neural lineages are specified by different mechanisms. While the A-line neural identity is specified cell autonomously, both a-line and b-line cells need to be induced to form neural tissue (Fig. 5).

Autonomously Forming CNS: The A-Line

The A4.1 blastomere will produce neural tissue when cultured in isolation from the 8-cell stage. This suggests that the A-line contains all the information required to develop neural fates (Minokawa *et al.*, 2001; Okada *et al.*, 1997). The A7.4 and A7.8 blastomeres of 44-cell embryos mainly give rise to neural tissue, while their sister blastomeres, A7.3 and A7.7, form notochord. Isolated A7.4 and A7.8 blastomeres will go on to express neural marker genes (Minokawa *et al.*, 2001). By contrast, the adoption of notochord fate in A7.3 and A7.7 depends on an inductive interaction with the underlying endoderm blastomeres (Nakatani and Nishida, 1994). This induction occurs between the 32- and 64-cell stages and is dependent on FGF/ras/MEK and BMP signalling (Darras and Nishida, 2001a; Kim and Nishida, 2001; Minokawa *et al.*, 2001; Nakatani and Nishida, 1997; Shimauchi *et al.*, 2001). In the absence of inductive signal, A7.3 and A7.7 adopt a neural fate. Conversely, if the notochord/neural precursor is isolated and treated with FGF, both daughters will assume a notochord fate (Minokawa *et al.*, 2001; Nakatani *et al.*, 1996). Therefore, at the 44-cell stage, the A-line notochord and neural precursors have identical potentials: their default fate is neural, their induced fate is notochord.

Acquisition of the neural fate does not require signals before the 44-cell stage either: when fertilised eggs, or isolated A4.1 blastomeres, were cultured in the absence of calcium, leading to cell dispersion, some cells still adopted a neural fate as assayed by the expression of the pan-neural marker ETR-1 (Minokawa *et al.*, 2001). This shows that neural fate specification, at least in some A-line cells, is a cell-autonomous process (Fig. 5).

A-line blastomeres give rise to both neurones and ependymal cells. To test whether neuronal differentiation was also achieved cell autonomously, A4.1 blastomere explants were cultured in conditions where cell contacts were impaired to a sufficient level to substantially block notochord induction. Under these conditions, expression of the neuronal sodium channel TuNa1 continued unperturbed (Okada *et al.*, 1997). Thus, it appears that neuronal differentiation in the A-line neural cells may also be an autonomous process. This result is further strengthened by experiments using isolated cleavage-arrested A4.1 blastomeres, which showed that cell–cell interactions are not required for neuronal differentiation (Okada *et al.*, 1997; Okamura *et al.*, 1997). Testing whether ependymal cells can also differentiate in the absence of cell interactions has so far been hampered by the lack of specific markers.

Altogether, these experiments indicate that the adoption of neural fates in the A-line involves a binary decision between notochord and neural fates, the latter fate forming in the absence of notochord-inducing signals. Because these experiments were done with general markers, they do not rule out that signalling events could be required for the specification of specific cell types, or for the regionalisation, of the A-line-derived CNS.

Induced CNS: The a- and b-Lines

a-line induction starts during the cleavage stages. In contrast to the situation in the A-line neural cells, animal cells do not form neural tissue when isolated at the eight-cell stage. When animal cells are cultured in isolation, no morphologically recognisable sensory vesicle, pigment cells, or palps form in any of the many ascidians studied (*Ascidiella*, *Styela*, *Ascidia*, *Phallusia*, *Halocynthia*, and *Ciona*) (Chabry, 1887; Conklin, 1905; Hudson and Lemaire, 2001; Nishida and Satoh, 1989; Ortolani *et al.*, 1979; Reverberi and Minganti, 1946; Rose, 1939). Conversely, removal of a4.2 from embryos or any combination of blastomeres that did not include a4.2 resulted in partial embryos with no, or very little, morphologically recognisable neural tissue (Reverberi and Minganti, 1946).

More recent studies extended these results by looking at the expression of specific markers of CNS and PNS neurones. These markers include neuronal-type membrane excitability as well as expression of genes for the sodium channels TuNa-1 and -2 (CNS and PNS neurones). In spite of the small size of the embryos, membrane excitability measurement was made possible by the observation that, when cytokinesis is blocked at early stages by cytochalasin B, nuclear division continues and the arrested cells proceed to differentiate according to their lineage (Hirano *et al.*, 1984; Whittaker *et al.*, 1977). Membrane excitability could hence be measured in a single large cell of an embryo or an explant, which had been cleavage-arrested from the eight-cell to the larval stages. In whole embryos cleavage-arrested at the eight-cell stage, both epidermal-type and neuronal-type action potentials were detectable, depending on the analysed blastomere (Hirano *et al.*, 1984). Cells either adopted one fate or the other and no cases of mixed neuronal and epidermal types were observed (Hirano *et al.*, 1984; Okado and Takahashi, 1990a; Okado and Takahashi, 1990b). Isolated a4.2 developed as epidermis, expressing an epidermis-specific epitope, showing epidermal-type membrane excitability and secreting a tunic (Okado and Takahashi, 1990b). When coisolated with A4.1, a4.2 exhibited neuronal membrane excitability, expressed the *TuNa* genes, and lost the expression of the epidermal marker (Nagahora *et al.*, 2000; Okada *et al.*, 1997; Okado and Takahashi, 1988, 1990a,b; Okamura *et al.*, 1993, 1994). Interestingly, in these recombinates, formation of *TuNa1*-positive neurones in a4.2 was not always accompanied by

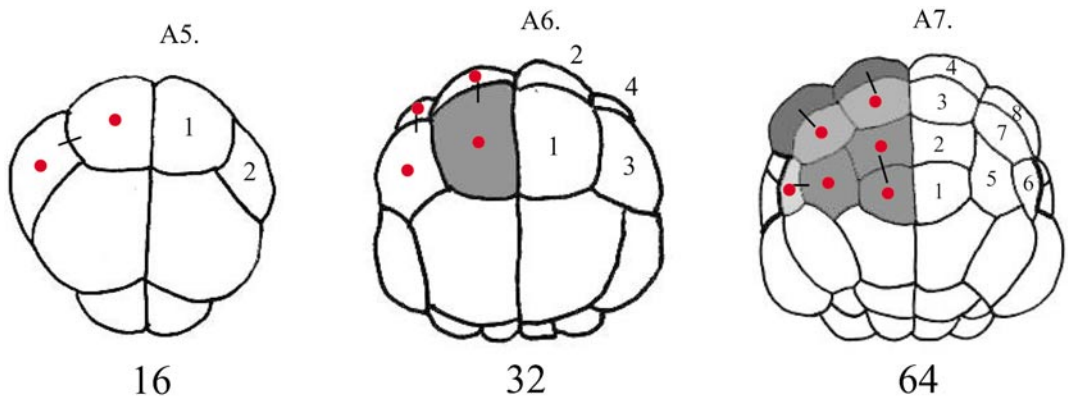

FIG. 6. Ability of the different A-blastomeres to induce pigment cells. Vegetal view of embryos at the 16-, 32-, and 64-cell stages; anterior is up. The blastomeres, which are fate-restricted, are in gray (very light gray for trunk lateral cells, light gray for notochord, dark gray for endoderm, and very dark gray for spinal cord). The blastomeres, which have the ability to induce pigment cells in isolated a4.2, are indicated by a red spot. For those blastomeres with inducing abilities, bars mark sister blastomeres.

expression of *TuNa1* in A4.1, suggesting that the interaction is not of homoeogenetic nature (Okada *et al.*, 1997). It is not known, however, whether neural tissue in the absence of neurone formation occurred in these cleavage-arrested A4.1 blatomeres. Taken together, these experiments established that the animal blastomeres absolutely require an interaction with the vegetal cells to differentiate into a morphologically identifiable sensory vesicle, including pigment cells and CNS/PNS neurones.

These results were confirmed in cleaving partial embryos by analysing, at the tailbud or larval stages, the expression of gelsolin (all PNS neurones), tyrosinase-related protein (pigment cell precursors), as well as an antibody against acetylated tubulin (some PNS and CNS neurones) (Darras and Nishida, 2001b; Hudson and Lemaire, 2001; Ohtsuka *et al.*, 2001a). The absence, in isolated a4.2 explants cultured to the tailbud stages, of the more general sensory vesicle or dorsoanterior epidermis markers *Otx*, *TBB2*, and *Gsx* suggests that indeed all a-line-derived neural structures require an inductive interaction (Darras and Nishida, 2001b; Hudson and Lemaire, 2001; Wada and Saiga, 1999b). Furthermore, this interaction was required before the end of gastrulation as expression of the general neural plate markers *Otx*, and *ETR-1* at this stage was undetectable in a4.2 blastomeres cultured in isolation from the eight-cell stage (Darras and Nishida, 2001b; Hudson and Lemaire, 2001).

Signalling between animal and vegetal blastomeres may actually start much earlier, at the time of the fate restriction of neural progenitors. In the a4.2 lineage, epidermal and neural plate fates start to be separated at the 32-cell stage: a6.5 is restricted to form anterior neural plate (sensory vesicle and palps) and a6.6 and a6.8 are restricted to form epidermis. The expression pattern of early neural and epidermal marker indicates that this fate restriction corresponds to the onset of distinct genetic programmes in the a6.5 and epidermal precursors (Ishida and Satoh, 1998; Wada *et al.*, 1996). At the onset of their expression, *Otx* and *EpiC*, an epidermis marker, show complementary expression. a6.5 expresses *Otx* but not *EpiC*. Conversely, a6.6 and a6.8 express *EpiC* but not *Otx*. This complementarity of *Otx* and *EpiC* expression persists in the a6.5 and a6.6/8 derivatives at the 64- and 110-cell stage. The onset of expression of *Otx* in the a6.5 neural precursor requires contact with vegetal blastomeres, indicating that an inductive interaction with the vegetal cells is required for the earliest steps of neural specification (Hudson and Lemaire, 2001).

Induction of the a-line neural fates is a multistep process. Analysis of the effect of the ablation of A-line or explantation of a-line blastomeres at different stages revealed that the acquisition of dorsoanterior epidermis and sensory vesicle fates in the neural plate progenitors is sequential.

In *H. roretzi*, when the animal pole is explanted at the 8- or 16-cell stage, *Dll1*, a palp marker, and *Otx* are not expressed at the tailbud stage (Wada *et al.*, 1999). Ablation of A4.1 at the 8-cell stage also prevented palp formation. The expression of *Otx* and *Dll1*, however, continued in animal pole explants isolated at the 32-cell stage. Ablation of A4.1 derivatives at the 32-cell stage also did not interfere with palp formation. In contrast, similar experiments revealed that no morphologically recognisable sensory vesicle or pigment cells form if the A-line is ablated at the 32-cell stage (Nishida and Satoh, 1989; Reverberi *et al.*, 1960; Wada *et al.*, 1999). Hence, a signal acting up to the 32-cell stage is sufficient to induce a palp fate, but not a morphologically recognisable sensory vesicle fate, in the common precursor of the palps and sensory vesicle. The palp and sensory vesicle fates separate at the 110-cell stage. When sensory vesicle precursors were isolated at this stage (Nishida, 1991), they did not express epidermal or palp markers, suggesting the acquisition of a sensory vesicle fate, for

which there were no general markers at the time this experiment was carried out. Consistent with the idea that acquisition of a sensory vesicle fate requires prolonged signalling from vegetal blastomeres, cleavage-arrested A4.1 and a4.2 needed to be in contact until at least the early gastrula stage to obtain neuronal-type excitability (Okado and Takahashi, 1990b).

Taken together, these experiments suggest at least two steps in the specification of sensory vesicle fates. Exposure of the a-line blastomeres to a neural inducer from the 16- to 32-cell stages converts a basal epidermal identity to a palp/dorsoanterior epidermis character in a6.5. Exposure to a further signal between the 32- and 110-cell is required to induce sensory vesicle identity in a8.17/19/25. It is at present unclear whether the first and second phases involve distinct inducing molecules. An interesting observation is that, each time, the timing of induction is intimately related to the timing of fate restriction. Neural plate and epidermal fates segregate at the 32-cell stage, while palps and sensory vesicle fates become restricted at the 110-cell stage.

Whether the first inductive event is a prerequirement for the acquisition of a sensory vesicle fate is still uncertain. In experiments with cleavage-arrested blastomeres, contact between a4.2 and A4.1 from the 110-cell stage onwards was sufficient to induce neuronal-type excitability, revealing that cleavage-arrested a4.2 that had not been subjected to an early induction at the 32-cell stage could still be induced to form neurones (Okado and Takahashi, 1990b). It remains to be seen whether this applies to more physiological experimental designs, and whether this does not simply mirror a temporal displacement of both phases of induction.

Regionalisation and diversification within a-line sensory vesicle precursors. Further inductive interactions are needed during gastrulation to obtain the different sensory vesicle cell types. For example, the induction of neuronal-type excitability, though initiated before gastrulation, continues during this process (Okado and Takahashi, 1990a). An elegant example is also provided by the study of pigment cell formation.

At the 110-cell stage, the precursors of the pigment cells (a8.25) are not yet restricted to a pigment cell fate and will also give rise to other sensory vesicle derivatives (Nishida, 1987). That they are not yet committed was shown by the failure of isolated a8.25 to develop melanin granules or tyrosinase activity at the larval stage (Nishida, 1991). Pigment cells become committed around the midgastrula stage (180 cells) as a result of an inductive process that takes place during gastrulation (Nishida, 1991; Nishida and Satoh, 1989). This commitment probably corresponds to the fate restriction of the a9.49 cell to a tyrosinase positive lineage (Sato *et al.*, 1999). In *Halocynthia*, this late inducing signal is emitted by the progeny of the A-line neural cells (A7.4 and A7.8) (Nishida, 1991). Interestingly, this signal is not required for the acquisition of a general neural identity by the a-line cells: bilateral ablation of A7.4 and A7.8 does not affect expression of the pan-neural marker ETR-1 while causing the absence of tyrosinase-positive cells (Darras and Nishida, 2001b).

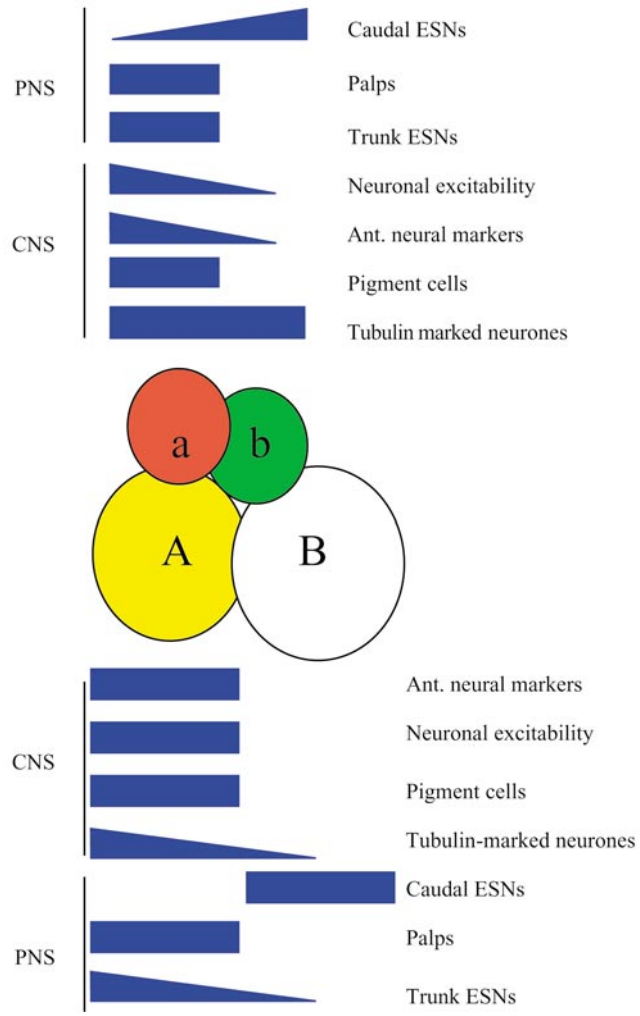

FIG. 7. Summary of differences in competence and inductive abilities in ascidian embryos. The inductive ability of vegetal cells to induce the fates indicated is shown below the vegetal cells. Wedges mark the relative inductive capacities. A thick wedge indicates high ability, a thin wedge little ability. Where no wedge is seen under a blastomere, this indicates that inductive ability is not detectable in that blastomere. The competence of animals to respond to these signals and form the fates indicated is shown above the animal cells. A box covering both blastomeres indicates they are equally competent. A wedge indicates relative competence. A thick wedge indicates high competence, a thin wedge little competence. Where no wedge is seen above a blastomere, this indicates that this cell was not competent to form this particular fate. Neural fates of both CNS and PNS are indicated. ESN, epidermal sensory neurones; a, a4.2; b, b4.2; A, A4.1; B, B4.1.

In summary, the available data suggest a model whereby at least three distinct steps can be identified in the generation of a patterned neural plate. In a first step, between the

16- and 32-cell stages, the a6.5 blastomeres lose their basal epidermal fate and acquire a palp/anterior–epidermis fate. In a second step, between the 32- and 110-cell stages, the sensory vesicle precursors acquire a sensory vesicle fate. Finally, during gastrulation, in a third step, which is more related to neurogenesis and regionalisation than to neural induction, neurones and pigment cells are specified. Each of these three phases require an interaction with the A-line blastomeres. The precise identity of the blastomeres that secrete the signals responsible, before gastrulation, for the successive induction of palps and sensory vesicle fate is currently unknown, but it seems that the initial neural inducing activity is broadly distributed in A-line cells (Reverberi *et al.*, 1960) (Fig. 6). These authors showed that, at the 16-cell stage, both A4.1 derivatives, A5.1 and A5.2, are able to induce pigment cells when recombined with a4.2. At the 32-cell stage, the endoderm precursor (A6.1), the endoderm-TLC precursor (A6.3), and both notochord-spinal cord precursors (A6.2/4) have the ability to induce pigment cells in a4.2. At the 64-cell stage, the ability to induce pigment cells becomes restricted to the endoderm precursors (A7.1/2/5), notochord precursors (A7.3/7), and TLC precursor (A7.6). The spinal cord precursors (A7.4/8) are not able to induce pigment cells in a4.2, though Nishida showed that they could induce presumptive sensory vesicle blastomeres of 110-cell embryos to adopt a pigment cell fate (Nishida, 1991). Assuming that the pigment cell induction reflects the sequential induction of a sensory vesicle identity followed by a pigment cell identity, these results suggest that at the progeny of each A-line cell of 16- to 32-cell stage embryos can send signals that induce both an initial sensory vesicle fate and a pigment cell fate. By the 64-cell stage, a separation of the signals has occurred and the progeny of the A-line neural precursors is no longer able to induce epidermis to form neural tissue but retains the ability to regionalise induced neural tissue (Darras and Nishida, 2001b; Nishida, 1991; Reverberi *et al.*, 1960).

The b-line. Cultured alone, b4.2 explants, like a4.2 explants, form balls of epidermis, elicit epidermal-type action potentials, express epidermal markers, and secrete tunic (Hudson and Lemaire, 2001; Okado and Takahashi, 1988, 1990b). In intact larvae, anti-acetylated tubulin antibodies mark a row of axonal-like structures running along the dorsal tail nerve cord (Crowther and Whittaker, 1992; Hudson and Lemaire, 2001; Nakatani *et al.*, 1999). Their dorsal position and lack of differentiation in A-line cells cultured in isolation (Hudson and Lemaire, 2001) suggest that these cells are derived from the b-lineage and may correspond to the dorsal peripheral axones (Crowther and Whittaker, 1992; Katz, 1983).

Anti-acetylated tubulin-positive neurones do not form in isolated b4.2 explants. They can, however, be induced by recombination with vegetal blastomeres (Hudson and Lemaire, 2001). The timing of this induction is currently unknown. Early specific markers of the neural b-line will be required to further study the adoption of a central neural fate in this lineage.

The b-line tail epidermal sensory neurones, found spaced at regular intervals along the tail, are marked by anti-acetylated tubulin and anti-gelsolin antibodies (Crowther and Whittaker, 1992; Hudson and Lemaire, 2001; Ohtsuka *et al.*, 2001b). These structures form in b-line cells only after inductive interaction with the vegetal cell, B4.1 (Hudson and Lemaire, 2001; Ohtsuka *et al.*, 2001a).

ESTABLISHMENT OF THE ANTEROPOSTERIOR IDENTITY OF THE INDUCED NERVOUS SYSTEM

The previous sections established that both b-line and a-line animal cells are induced to form nervous tissues. An important issue is how the two induced lineages come to form very different types of neural structures. Several experiments have addressed whether a- and b-line cells show differential competence to vegetal signals and whether vegetal blastomeres of the A- and B-line secrete different neural inducers.

Differential Competence in Animal Hemisphere Neural Cells

Blastomere recombination or ablation experiments show that, although b4.2 can be induced to form pigment cells, it does so much less readily than a4.2 (Hudson and Lemaire, 2001; Nishida and Satoh, 1989; Rose, 1939). Similarly, when the excitability of membranes was tested in cleavage arrested animal cells isolated from 8- or 16-cell embryos, it was found that b4.2 can form neuronal-type action potentials when in contact with A4.1, but to a lesser degree than a4.2 (Okado and Takahashi, 1990b, 1993). a4.2 explants also showed a markedly higher competence than b4.2 to A4.1-mediated induction of the sensory vesicle markers gsx and *Otx* (Hudson and Lemaire, 2001). This result showed that there was a difference in competence, not only for specific neuronal cell types, such as pigment cells and neurones, but also for more general markers of a4.2 neural fates.

Interestingly, b4.2 can adopt a neural fate in response to signals from A4.1 and B4.1, but the neural fate adopted is different to that of induced a4.2 explants (Fig. 7). Induced b4.2 explants develop acetylated tubulin-positive neurones in a similar way as a4.2 explants, but do not express anterior marker genes (Hudson and Lemaire, 2001). Additionally, b4.2 can form tail peripheral ciliated epidermal sensory neurones much more readily than a4.2 (Hudson and Lemaire, 2001; Ohtsuka *et al.*, 2001a). Altogether, therefore, a4.2 and b4.2 can both respond to signals emitted from the vegetal cells, but the output fates markedly differ. This difference can already be detected at the gastrula stages (Hudson and Lemaire, 2001). These data are summarised in Fig. 7.

A difference in competence is also seen within the a-line at the 16-cell stage. Okado and Takahashi (1993) compared the competence of a5.4, a5.3, b5.4, and b5.3, daughter cells of a4.2 and b4.2, to exhibit neuronal-type excitability in response to vegetal signals. They found that a5.3 was more receptive than a5.4, which was more receptive than b5.3 and b5.4, which behaved similarly. They proposed that the competence of cells to express a neural fate in response to the natural inducer A4.1 was proportional to the amount of neural tissue in a larva to which the respective blastomere normally contributes. This led the authors to propose a model of neural induction in which the inductive signal is permissive rather than instructive, simply revealing a natural inclination of some blastomeres to form neural tissue. The fact that b5.4 forms no neural tissue *in vivo*, although it is as competent as b5.3 to respond to neural induction, seems at odds with this proposition. This was interpreted as an indication that b5.4 inherited the same neural determinant(s) as b5.3, but that neural formation was not revealed during normal development because b5.4 does not come into contact with A4.1 derivatives. Thus, in this case, it is the topology of the embryo that leads to regional specificity.

The molecular basis for the differential competence of animal blastomeres remains an open issue. The fact that a4.2 and b4.2 have already acquired different competences at the eight-cell stage, that is before or at the onset of zygotic transcription, strongly suggests that the inheritance of different maternal determinants plays a key role in the process. Evidence for the existence of a maternal determinant of posterior identities comes from studies removing regions of cytoplasm in the fertilised eggs of *Halocynthia* (Nishida, 1994). Removing or transplanting anterior vegetal cytoplasm had little effect on embryo patterning. However, removal of a region of coloured cytoplasm, which is localised to the future posterior pole of the embryo during a process termed ooplasmic segregation, has a profound effect. The establishment of the initial AP polarity of the embryo leads to different cleavage patterns during the cleavage stages. Embryos depleted of their posterior cytoplasm appear radialised with only anterior cleavage patterns. This is accompanied by a transformation of B4.1-derived posterior vegetal fates into A4.1-like anterior fates. Conversely, transplanting the posterior plasm to the future anterior side of a normal embryo suppresses anterior development and extends posterior fates. These results showed that, at least for the vegetal cells, dominant posterior determinants act to repress a default anterior fate. The fate of the animals cells was not assayed in these experiments other than showing that epidermal development was not suppressed. Are b4.2 fates transformed to a4.2-like fates, in a similar way to the vegetal cell transformations? Pigment cells or palps are not apparent in the radialised anterior embryos, but this may be due to the severe morphological defects that occur in these manipulated embryos. It would be very interesting to look at expression of *ETR-1*, *Otx*, and regional anterior neural markers such as *TRP* or *Gsx* in early posterior plasm-depleted embryos to see if they are now expressed in b-line territories. Should this be the case, it will be interesting to test candidate molecules expressed in the posterior plasm. One of these is the recently isolated Zic-like maternal transcription factor Macho-1, which has been shown to encode a critical determinant of muscle, the major posterior vegetal fate (Nishida and Sawada, 2001). It will also be interesting to analyse whether the maternal competence factors act directly on the regulation of anterior neural genes, or via intermediate zygotic competence factors.

The Vegetal Blastomeres A4.1 and B4.1 Have Different Inducing Capacities

As discussed above, the progenies of a4.2 and b4.2 differ in their competence to respond to vegetal signals. Conversely, several authors analysed whether vegetal blastomeres of the A- and B-lines also differed in their signalling abilities. Ablation of A4.1 led to the loss of sensory vesicle and palps, suggesting that B4.1 was not able to compensate for the loss of A4.1. Consistently, when B4.1 was recombined with a4.2, no induction of anterior neural tissue occurred (Hudson and Lemaire, 2001; Okado and Takahashi, 1990b; Reverberi and Minganti, 1947). This contrasts with the finding that progeny of A4.1 and B4.1 are interchangeable with regard to mesoderm-inducing signals (Kim *et al.*, 2000). While these experiments indicate that the progeny of A4.1 and B4.1 behave differently with respect to neural induction, they do not rule out that some progeny of B4.1 can at some time in their history also secrete inducers of anterior neural fates. Heterochronic recombinations between B4.1 and a4.2 derivatives can lead to the induction of anterior neural fates in the animal cells (Whittaker, 1987). The relevance of this finding for normal development awaits further experiments.

On the basis of the morphological effect of the ablation of B4.1 or b4.2, several authors also proposed that posterior blastomeres exert a negative influence on anterior neural fates and especially on pigment cell formation (Reverberi and Minganti, 1946, 1947; Rose, 1939). However, this issue should be addressed again with the new molecular markers made available by the recent *in situ* hybridisation screens (Satou *et al.*, 2001c).

While the progeny of A4.1 appears to emit a more potent inducer of anterior neural fates than that of B4.1, the B4.1 lineage is a much more potent inducer of the posterior ciliated epidermal sensory neurones than A4.1 (Hudson and Lemaire, 2001; Ohtsuka *et al.*, 2001a). Thus, different signals may be emitted from A4.1 and B4.1 for the induction of these different types of neurones (Fig. 7). It is at present unclear whether this difference reflects a qualitative, quantitative, or temporal difference. The molecular identification of the inducing signals should shed light on this issue. It will also be of interest to assay whether posterior plasm or Macho1-depleted B4.1-line display A4.1-like inducing properties.

In summary, the data reported in this section establish

that the regional identity of the induced CNS and PNS lineages is achieved by a tight spatial regulation of both the competence of a- and b-line animal blastomeres to respond to vegetal signals and of the signals emitted by A- and B-line vegetal blastomeres.

MOLECULAR NATURE OF THE INDUCER(S)

The inducer(s) described in the previous sections could be either secreted or transmembrane proteins, or small molecules diffusing via gap junctions. Experiments with the secretion inhibitors Monensin, brefeldin A, and bafilomycin A1 have shown that the induction of neuronal-type excitability needs an intact secretory pathway, suggesting that at least some of the inducer(s) are either secreted or transmembrane molecules (Okado and Takahashi, 1993). Several candidate pathways have recently emerged.

Proteases

Historically, the first molecule with reported neural-inducing activity was the serine protease trypsin, which was shown to induce pigment cells in naive animal caps (Ortolani *et al.*, 1979). These findings were extended by showing that serine proteases could also elicit neuronal differentiation (Okado and Takahashi, 1990b). These authors further tested the activity of several types of serine proteases and suggested that a Subtilisin-like protease may constitute a good candidate inducer (Okado and Takahashi, 1993). In addition, they showed that two inhibitors of serine proteases decreased the induction of neural excitability in A4.1/a4.2 cleavage-arrested embryos: SSI (Streptomyces subtilisin inhibitor), a specific inhibitor of subtilisin-type proteases, and a2-macro-globulin, a broad-spectrum inhibitor. Induction was suppressed at most in 50% of recombinates. The partial effect may be due to the inability of SSI to penetrate the tight membrane space between animal and vegetal blastomeres, to the existence of other proteases not blocked by the inhibitors, or to the involvement of additional types of signalling molecules during the induction process.

While the above experiments implicate subtilisin-like proteases in the neural induction process, their precise relationship to the A4.1-derived signal remains unclear. The timing of loss of competence to develop neuronal excitability of cleavage-arrested a4.2 to pronase and to signals coming from A4.1 was indistinguishable. The onset of competence, however, differed. Competence to respond to pronase started at the 8-cell stage, while competence to respond to A4.1 was first detected at the 64-cell stage. Likewise, while a 15-min treatment with pronase during the cleavage stages can induce a neural fate (neuronal-type excitability), several hours of contact with A4.1 are required to obtain the same effect (Okado and Takahashi, 1990b). This latter difference, however, could be due to the inefficient washing of the pronase. Finally, pronase and subtilisin appeared to be better inducers than A4.1 of a neural fate in b-line derivatives (Okado and Takahashi, 1990b, 1993).

Several serine proteases have been identified in the EST screen carried out in Japan. Some of these ESTs were obtained from egg or cleavage-stage cDNA libraries, suggesting that these proteases may be present in the embryo at the time of neural induction. The precise expression patterns of the corresponding genes, however, has not yet been determined.

FGFs

Treatment of a4.2 with 1–100 ng/ml of recombinant bFGF led to the induction of neural-type excitability in *Halocynthia* (Inazawa *et al.*, 1998). Analysis of the expression of molecular markers in *Ciona* and *Halocynthia* confirmed the neural-inducing ability of FGF and indicated that it can induce both sensory vesicle and dorsoanterior epidermis fates in a4.2 explants (Darras and Nishida, 2001b; Hudson and Lemaire, 2001). This induction was probably direct as no tissue other than epidermis and neural tissue was detected in the treated explants. Treatment of *Halocynthia* a4.2 cells between 16- and 32-cell stage did not allow the formation of sensory vesicle, while treatment from the 16- to the 64-cell stage did (Darras and Nishida, 2001b). Interestingly, even longer treatments with FGF could not entirely mimic the A4.1-derived signals, as the posterior sensory vesicle marker *Gsx* was never induced at the tailbud stage (Hudson and Lemaire, 2001).

The temporal competence of cleavage-arrested a4.2 to respond to FGF or the A4.1-derived signal and show neuronal excitability was indistinguishable (Inazawa *et al.*, 1998). Likewise, no difference was found in the duration of the signalling event required for the induction of neuronal fates by FGF or A4.1 (Inazawa *et al.*, 1998). In addition, FGF, like the A4.1-derived signal, could induce anterior neural fates in a4.2 but not in b4.2 at both the gastrula and tailbud stages (Hudson and Lemaire, 2001).

Evidence from loss-of-function studies also supports a role for FGF in the induction of anterior neural fates. Using various inhibitors for the FGF/ras/MEK pathway, Kim and Nishida (2001) demonstrated a requirement for this pathway for the expression of the neural marker *ETR-1* at the neurula stage. This study established that the endogenous FGF signal acted before the 64-cell stage, a finding consistent with the induction of *ETR-1* in response to a treatment of a4.2 with bFGF from the 16- to the 64-cell stage (Darras and Nishida, 2001b). It remains, however, unclear from this study whether FGF signalling is acting directly on the neural precursors. Indeed, the notochord, which can induce neural fates in animal cells (Reverberi *et al.*, 1960), is itself induced by an FGF signal before the 64-cell stage (Darras and Nishida, 2001a; Kim *et al.*, 2000; Nakatani *et al.*, 1996). The inhibition of FGF/MEK signalling throughout the embryo could thus not discriminate between a direct effect of FGF signalling on neural induction or an indirect effect via

the induction of the notochord. The best arguments for an early direct effect of FGF therefore remain the very rapid activation by FGF of *Otx* in cleavage-stage animal explants and the fact that expression of this gene during normal development is initiated before completion of notochord induction (Hudson and Lemaire, 2001).

Finally, in addition to being an inducer of anterior neural fates, FGF, like vegetal blastomeres, is also able to induce peripheral ESNs in both a4.2 and b4.2 (Hudson and Lemaire, 2001; Ohtsuka *et al.*, 2001a). The competence of animal cells to form ESNs in response to FGF is lost by the neurula stages (Ohtsuka *et al.*, 2001a). The presence of ESNs in embryos deficient for FGF signalling has not been analysed.

Which FGF could be implicated? All the experiments carried out so far involved the treatment of ascidian explants with recombinant mammalian FGF-1 (aFGF) or FGF-2 (bFGF). As in other systems, these two FGFs have different effects: in contrast to bFGF, aFGF was not able to induce neural-type excitability in cleavage arrested a4.2 (Inazawa *et al.*, 1998). At least six different FGFs can be identified by searching the results of the different genomic or EST programmes (Satou *et al.*, 2002). One of these factors, FGF 9/16/20, a direct target of β-catenin, is expressed in the A-line blastomeres during cleavage stages (Imai *et al.*, 2002). Their expression patterns during the cleavage stages, as well as the effects of their over- or underexpression, remain to be determined. Identification of the ascidian FGF(s) involved in the induction process may also help determine the range of diffusion of FGFs in ascidians. The very local nature of the inductive process suggests that, instead of being freely diffusible as in vertebrates, they may remain closely associated to the membranes of the secreting cells.

BMP Antagonists

Work in vertebrates has suggested that inhibition of BMP signalling is a central event during neural induction (Harland, 2000). The model known as the "default model for neural induction" proposes that, in lower vertebrates, ectodermal cells only adopt an epidermal fate if they receive a BMP2/4 signal. Inhibition of this signal, by either cell dispersion or the antagonism of BMP proteins by secreted inhibitors, such as Chordin, lead to the adoption of a default neural fate. Overexpression of *Bmpb*, the ascidian homologue of *Bmp2/4*, led to a repression of the palp and pigment cell fates, suggesting that BMPs may have a similar role in ascidians and vertebrates (Miya *et al.*, 1997). Analysis of a larger panel of markers and of the effect of varying concentrations of BMPb or its antagonist Chordin suggested, however, a different interpretation (Darras and Nishida, 2001b). These authors confirmed that *Bmpb* overexpression antagonised palps formation, but found no evidence for an adverse effect at the tailbud stage on the neural marker *ETR-1*. Conversely, overexpression of *Chordin* prevented the separation of the three palps, leading to a large single palp. It did not lead to a down-regulation of the pan-epidermal marker *EpiC*, nor to an extension of the *ETR-1*-positive neural territories. Taken together, these results suggest that the BMP/Chordin antagonism is not a major player in neural induction, though it appears to be involved in palp differentiation or specification. Also consistent with the idea that neural is not the default fate of ascidian animal cells, their dispersion during the cleavage stages is insufficient to activate the expression of *ETR-1* (Minokawa *et al.*, 2001).

While BMP/Chordin may not play a significant role during neural induction, it plays a later role in the specification of pigment cells at the beginning of gastrulation (Darras and Nishida, 2001b). At the 110-cell stage, *Bmpb* is expressed in the A8.15 and A8.16 A-line neural precursors, which directly contact the pigment cell precursor a8.25. Conversely, *Chordin* is expressed in the a8.26 rostral trunk epidermal neurones (RTEN) precursor. Overexpression of *Chordin* leads to loss of pigment cells, while moderate overexpression of *Bmpb* leads to the formation of additional pigment cells. BMPb therefore appears to constitute a prime candidate factor for the third step of pigment cell induction. This is further strengthened by the demonstration that BMPb complements FGF to induce pigment cells in naive a4.2 blastomeres.

In conclusion of this section, at least three pathways seem to be involved in ascidian early neural tissue formation: subtilisin-like proteases, FGF, and BMPs. While the latter seems to act later than the two other pathways, the epistatic relationship of FGF and proteases needs to be addressed. Also, the rapid expansion of the list of pathways implicated in vertebrate neural induction suggests that, in ascidians as well, we only have a partial view of the process. Recently, factors of the IGF family were shown to play a role in neural induction in *Xenopus* (Pera *et al.*, 2001), while inhibition of Wnt signalling is important for anterior neural specification in *Xenopus* (Niehrs, 1999) and for neural induction in amniotes (Wilson and Edlund, 2001). The ascidian genome contains an IGF1 Receptor, as well as several Wnts or inhibitors of Wnt signalling. It will be important to test their role in the induction process. Retinoic acid (RA) also probably plays a role in the regionalisation of the anterior neural plate as RA-treated embryos have impaired palps and pharynx (Hinman and Degnan, 1998, 2000; Katsuyama and Saiga, 1998; Katsuyama *et al.*, 1995). In contrast, TGFβ1, ActivinA, and EGF had no effect on the default epidermal fate of a4.2 blastomere (Inazawa *et al.*, 1998).

CONCLUSION AND PERSPECTIVES

The availability of a larger panel of molecular markers has recently enabled significant advances in our understanding of the early steps of nervous system formation. These advances have revealed features common to protochordates and vertebrates but also mechanisms that strikingly differ.

The use of very similar strategies in phylogenetically distant animals suggests an ancestral use at the emergence of the chordates. Comparison of the strategies and pathways used in ascidians and vertebrates in the early steps of neural tissue formation hence suggests that induction of the nervous system by an FGF-like factor is an ancestral strategy used by the last common ancestor of vertebrates and ascidians. Likewise, the sequential specification of dorsoanterior epidermis and sensory vesicle is similar to the progressive specification of anterior epidermis (cement gland) and brain in *Xenopus* (Sive *et al.*, 1989).

Conversely, the use of divergent strategies suggests that either ascidians or vertebrates have acquired specialised strategies during evolution. One apparent difference is the existence in ascidians of a common precursor for posterior neural and notochord cells in the A-line. This seems to contradict the accepted model of neural induction in vertebrates, in which a binary decision is achieved between epidermal and neural fates. There is, however, evidence for common precursors for notochord and nerve cord cells in the chick (Selleck and Stern, 1991). Likewise, in fish and mice, interference with nodal signalling transforms cells normally fated to form axial mesoderm into neural tissue (Lu *et al.*, 2001; Schier and Talbot, 2001). The discrepancy between ascidians and vertebrates may thus be more superficial than initially thought, and it will be interesting to compare in vertebrates and ascidians the mechanisms of the binary decision between mesoderm and neural tissue.

A perhaps more fundamental difference is found when analysing the role of the BMP pathway in vertebrate and ascidian neural specification. On the basis of a common requirement for neural fates to form in *Drosophila* and vertebrates, inhibition of BMP signalling has been proposed to be a conserved strategy used by the ancestral bilateria, *Urbilateria* (DeRobertis and Sasai, 1996). Yet, ascidians do not seem to need inhibition of BMP signalling for neural tissues to form. The BMP pathway appears to be used to in amniotes to maintain an initial neural identity specified by FGFs (Wilson and Edlund, 2001). In ascidians, cell fates are determined much earlier than in vertebrates. The palp fate is determined by the 32-cell stage, while a sensory vesicle fate is likely to be formed by the 110-cell stage. This early determination may have rendered dispensable the maintenance function assured by the Chordin/BMP system. It is interesting in this respect to note that later functions of BMPs in the specification of dorsal neural fates seem to have been conserved (Darras and Nishida, 2001b).

Finally, the great progress achieved in the past 5 years has left large areas unexplored. We now have a good conceptual canvas, but we still know remarkably little about the molecular identity of the inducing factors, of the competence factors, and of the factors that occur upstream or downstream of the inductive process. For example, the signals involved may equally be of maternal or zygotic origin. β-Catenin probably plays an important function in the early cleavage stages as interference with β-catenin function leads to an expansion of epidermal territories (Imai *et al.*, 2000). It will be interesting to test whether this is at the expense of neural, mesodermal, or endodermal fates. No gene has been so far characterised as being a direct target of the neural inducer. The early activation of *Otx* and repression of *EpiC* suggest that these genes are prime candidates. The function of *Otx* in *Ciona* has been assessed by injection of antisense morpholino oligonucleotides (Satou *et al.*, 2001b) as well as by RNA injection (Wada and Saiga, 1999b). Overexpression of *Otx* in isolated animal cells leads to a suppression of the epidermal fate and an expansion of the domain of expression of two marker genes: tubulin (*TBB2*) and the tyrosinase-related protein (*TRP*). Conversely, injection of *Otx* morpholinos leads to a loss of melanised pigment cells and palps. These are early, partial results, but they point the way.

The near future will likely see the identification and functional dissection of more early targets of the inducers as well as the targets of the targets, etc.. The combination of a sequenced genome, of large-scale *in situ* hybridisation screens, and of powerful methods to analyse gene function and regulation will no doubt help in this task and soon lead to the unravelling of the genetic cascade that leads to a simple patterned chordate neural plate.

ACKNOWLEDGMENTS

We thank S. Moody for initially encouraging us to write this review. We also thank Dr. Y. Okamura, U. Rothbächer, L. Kodjabachian, and S. Darras for discussions and comments on the manuscript. Our thanks also go to E. Lewis, C. Waldmann, and P. Weber for help with figures. Work in our laboratory is supported by the CNRS, the French Ministry of Research (ACI programme), the ARC, the AFM, the HFSPO, and the European Community.

REFERENCES

Aniello, F., Locascio, A., Villani, M. G., Di Gregorio, A., Fucci, L., and Branno, M. (1999). Identification and developmental expression of Ci-msxb: A novel homologue of Drosophila msh gene in Ciona intestinalis. *Mech. Dev.* **88,** 123–126.

Bone, Q., and Ryan, K. P. (1978). Cupular sense organs in Ciona (Tunicata: ascidiacea). *J. Zool. Lond.* **186,** 417–429.

Chabry, L. (1887). Embryologie normale et tératologique des Ascidies simples. *J. Anat. Physiol. (Paris)* **23,** 167–319.

Conklin, E. G. (1905). The organisation and cell lineage of the ascidian egg. *J. Acad. Natl. Sci. (Philadelphia)* **13,** 1–119.

Corbo, J. C., Di Gregorio, A., and Levine, M. (2001). The ascidian as a model organism in developmental and evolutionary biology. *Cell* **106,** 535–538.

Corbo, J. C., Erives, A., Di Gregorio, A., Chang, A., and Levine, M. (1997a). Dorsoventral patterning of the vertebrate neural tube is conserved in a protochordate. *Development* **124,** 2335–2344.

Corbo, J. C., Levine, M., and Zeller, R. W. (1997b). Characterization of a notochord-specific enhancer from the Brachyury promoter region of the ascidian, *Ciona intestinalis*. *Development* **124,** 589–602.

Crowther, R. J., and Whittaker, J. R. (1992). Structure of the caudal neural tube in an ascidian larva: Vestiges of its possible evolutionary origin from a ciliated band. *J. Neurobiol.* **23,** 280–292.

Darras, S., and Nishida, H. (2001a). The BMP signaling pathway is required together with the FGF pathway for notochord induction in the ascidian embryo. *Development* **128,** 2629–2638.

Darras, S., and Nishida, H. (2001b). The BMP/CHORDIN antagonism controls sensory pigment cell specification and differentiation in the ascidian embryo. *Dev. Biol.* **236,** 271–288.

DeRobertis, E. M., and Sasai, Y. (1996). A common plan for dorsoventral patterning in Bilateria. *Nature* **380,** 37–40.

Di Gregorio, A., Spagnuolo, A., Ristoratore, F., Pischetola, M., Aniello, F., Branno, M., Cariello, L., and Di Lauro, R. (1995). Cloning of ascidian homeobox genes provides evidence for a primordial chordate cluster. *Gene* **156,** 253–257.

Gilbert, S. F. (1997). "Developmental Biology." Sinauer Associates, Inc., Sunderland, Massachusetts.

Gionti, M., Ristoratore, F., Di Gregorio, A., Aniello, F., Branno, M., and Di Lauro, R. (1998). Cihox5, a new Ciona intestinalis Hox-related gene, is involved in regionalization of the spinal cord. *Dev. Genes Evol.* **207,** 515–523.

Glardon, S., Callaerts, P., Halder, G., and Gehring, W. J. (1997). Conservation of Pax-6 in a lower chordate, the ascidian *Phallusia mammillata*. *Development* **124,** 817–825.

Harland, R. (2000). Neural induction. *Curr. Opin. Genet. Dev.* **10,** 357–362.

Hinman, V. F., and Degnan, B. M. (1998). Retinoic acid disrupts anterior ectodermal and endodermal development in ascidian larvae and postlarvae. *Dev. Genes Evol.* **208,** 336–345.

Hinman, V. F., and Degnan, B. M. (2000). Retinoic acid perturbs Otx gene expression in the ascidian pharynx. *Dev. Genes Evol.* **210,** 129–139.

Hirano, T., Takahashi, K., and Yamashita, N. (1984). Determination of excitability types in blastomeres of the cleavage-arrested but differentiated embryos of an ascidian. *J. Physiol.* **347,** 301–325.

Holland, L. Z., and Holland, N. D. (1999). Chordate origins of the vertebrate central nervous system. *Curr. Opin. Neurobiol.* **9,** 596–602.

Holland, L. Z., and Holland, N. D. (2001). Evolution of neural crest and placodes: Amphioxus as a model for the ancestral vertebrate? *J. Anat.* **199,** 85–98.

Hudson, C., and Lemaire, P. (2001). Induction of anterior neural fates in the ascidian *Ciona intestinalis*. *Mech. Dev.* **100,** 189–203.

Imai, K., Takada, N., Satoh, N., and Satou, Y. (2000). β-Catenin mediates the specification of endoderm cells in ascidian embryos. *Development* **127,** 3009–3020.

Imai, K., Satoh, N., and Satou, Y. (2002). Early embryonic expression of the FGF 4/6/9 gene and its role in the induction of mesenchyme and notochord in *Ciona saviguyi* embryos. *Development,* **129** 1729–1738.

Inazawa, T., Okamura, Y., and Takahashi, K. (1998). Basic fibroblast growth factor induction of neuronal ion channel expression in ascidian ectodermal blastomeres. *J. Physiol.* **511,** 347–359.

Ishida, K., and Satoh, N. (1998). Quantity of prelocalized maternal factor is associated with the timing of initiation of an epidermis-specific gene expression of the ascidian embryo. *Dev. Genes Evol.* **208,** 151–156.

Katsuyama, Y., and Saiga, H. (1998). Retinoic acid affects patterning along the anterior–posterior axis of the ascidian embryo. *Dev. Growth Differ.* **40,** 413–422.

Katsuyama, Y., Wada, S., Yasugi, S., and Saiga, H. (1995). Expression of the labial group Hox gene HrHox-1 and its alteration induced by retinoic acid in development of the ascidian *Halocynthia roretzi*. *Development* **121,** 3197–3205.

Katz, M. J. (1983). Comparative anatomy of the tunicate tadpole, *Ciona intestinalis*. *Biol. Bull.* **164,** 1–27.

Kim, G. J., and Nishida, H. (2001). Role of the FGF and MEK signaling pathway in the ascidian embryo. *Dev. Growth Differ.* **43,** 521–533.

Kim, G. J., Yamada, A., and Nishida, H. (2000). An FGF signal from endoderm and localized factors in the posterior–vegetal egg cytoplasm pattern the mesodermal tissues in the ascidian embryo. *Development* **127,** 2853–2862.

Locascio, A., Aniello, F., Amoroso, A., Manzanares, M., Krumlauf, R., and Branno, M. (1999). Patterning the ascidian nervous system: Structure, expression and transgenic analysis of the CiHox3 gene. *Development* **126,** 4737–4748.

Lu, C. C., Brennan, J., and Robertson, E. J. (2001). From fertilization to gastrulation: Axis formation in the mouse embryo. *Curr. Opin. Genet. Dev.* **11,** 384–392.

Manni, L., Lane, N. J., Sorrentino, M., Zaniolo, G., and Burighel, P. (1999). Mechanism of neurogenesis during the embryonic development of a tunicate. *J. Comp. Neurol.* **412,** 527–541.

Mayor, R., and Aybar, M. J. (2001). Induction and development of neural crest in *Xenopus laevis*. *Cell Tissue Res.* **305,** 203–209.

Meinertzhagen, I. A., and Okamura, Y. (2001). The larval ascidian nervous system: The chordate brain from its small beginnings. *Trends Neurosci.* **24,** 401–410.

Minokawa, T., Yagi, K., Makabe, K. W., and Nishida, H. (2001). Binary specification of nerve cord and notochord cell fates in ascidian embryos. *Development* **128,** 2007–2017.

Miya, T., Morita, K., Suzuki, A., Ueno, N., and Satoh, N. (1997). Functional analysis of an ascidian homologue of vertebrate Bmp-2/Bmp-4 suggests its role in the inhibition of neural fate specification. *Development* **124,** 5149–5159.

Moody, R., Davis, S. W., Cubas, F., and Smith, W. C. (1999). Isolation of developmental mutants of the ascidian *Ciona savignyi*. *Mol. Gen. Genet.* **262,** 199–206.

Munro, E. M., and Odell, G. M. (2002). Polarized basolateral cell motility underlies invagination and convergent extension of the ascidian notochord. *Development* **129,** 13–24.

Nagahora, H., Okada, T., Yahagi, N., Chong, J. A., Mandel, G., and Okamura, Y. (2000). Diversity of voltage-gated sodium channels in the ascidian larval nervous system. *Biochem. Biophys. Res. Commun.* **275,** 558–564.

Nakatani, Y., Moody, R., and Smith, W. C. (1999). Mutations affecting tail and notochord development in the ascidian *Ciona savignyi*. *Development* **126,** 3293–3301.

Nakatani, Y., and Nishida, H. (1994). Induction of notochord during ascidian embryogenesis. *Dev. Biol.* **166,** 289–299.

Nakatani, Y., and Nishida, H. (1997). Ras is an essential component for notochord formation during ascidian embryogenesis. *Mech. Dev.* **68,** 81–89.

Nakatani, Y., Yasuo, H., Satoh, N., and Nishida, H. (1996). Basic fibroblast growth factor induces notochord formation and the expression of As-T, a Brachyury homolog, during ascidian embryogenesis. *Development* **122,** 2023–2031.

Nicol, D., and Meinertzhagen, I. A. (1988a). Development of the central nervous system of the larva of the ascidian, *Ciona intestinalis* L. I. The early lineages of the neural plate. *Dev. Biol.* **130,** 721–736.

Nicol, D., and Meinertzhagen, I. A. (1988b). Development of the central nervous system of the larva of the ascidian, *Ciona*

intestinalis L. II. Neural plate morphogenesis and cell lineages during neurulation. *Dev. Biol.* **130,** 737–766.

Nicol, D., and Meinertzhagen, I. A. (1991). Cell counts and maps in the larval central nervous system of the ascidian *Ciona intestinalis* (L.). *J. Comp. Neurol.* **309,** 415–429.

Niehrs, C. (1999). Head in the WNT: The molecular nature of Spemann's head organizer. *Trends Genet.* **15,** 314–319.

Nishida, H. (1987). Cell lineage analysis in ascidian embryos by intracellular injection of a tracer enzyme. III. Up to the tissue restricted stage. *Dev. Biol.* **121,** 526–541.

Nishida, H. (1991). Induction of brain and sensory pigment cells in the ascidian embryo analyzed by experiments with isolated blastomeres. *Development* **112,** 389–395.

Nishida, H. (1994). Localization of determinants for formation of the anterior–posterior axis in eggs of the ascidian *Halocynthia roretzi*. *Development* **120,** 3093–3104.

Nishida, H., and Satoh, N. (1989). Determination and regulation in the pigment cell lineage of the ascidian embryo. *Dev. Biol.* **132,** 355–367.

Nishida, H., and Sawada, K. (2001). macho-1 encodes a localized mRNA in ascidian eggs that specifies muscle fate during embryogenesis. *Nature* **409,** 724–729.

Oda, I., and Saiga, H. (2001). Hremx, the ascidian homologue of ems/emx, is expressed in the anterior and posterior–lateral epidermis but not in the central nervous system during embryogenesis. *Dev. Genes Evol.* **211,** 291–298.

Ohtsuka, Y., Obinata, T., and Okamura, Y. (2001a). Induction of ascidian peripheral neuron by vegetal blastomeres. *Dev. Biol.* **239,** 107–117.

Ohtsuka, Y., Okamura, Y., and Obinata, T. (2001b). Changes in gelsolin expression during ascidian metamorphosis. *Dev. Genes Evol.* **211,** 252–256.

Okada, T., Hirano, H., Takahashi, K., and Okamura, Y. (1997). Distinct neuronal lineages of the ascidian embryo revealed by expression of a sodium channel gene. *Dev. Biol.* **190,** 257–272.

Okada, T., Katsuyama, Y., Ono, F., and Okamura, Y. (2002). The development of three identified motor neurons in the ascidian embryo. *Dev. Biol.*, **244,** 278–292.

Okado, H., and Takahashi, K. (1988). A simple "neural induction" model with two interacting cleavage-arrested ascidian blastomeres. *Proc. Natl. Acad. Sci. USA* **85,** 6197–6201.

Okado, H., and Takahashi, K. (1990a). Differentiation of membrane excitability in isolated cleavage-arrested blastomeres from early ascidian embryos. *J. Physiol.* **427,** 583–602.

Okado, H., and Takahashi, K. (1990b). Induced neural-type differentiation in the cleavage-arrested blastomere isolated from early ascidian embryos. *J. Physiol.* **427,** 603–623.

Okado, H., and Takahashi, K. (1993). Neural differentiation in cleavage-arrested ascidian blastomeres induced by a proteolytic enzyme. *J. Physiol.* **463,** 269–290.

Okamura, Y., Okado, H., and Takahashi, K. (1993). The ascidian embryo as a prototype of vertebrate neurogenesis. *Bioessays* **15,** 723–730.

Okamura, Y., Ono, F., and Okagaki, R. (1997). Regulation of voltage-gated ion channels during ascidian embryogenesis. *Dev. Neurosci.* **19,** 23–24.

Okamura, Y., Ono, F., Okagaki, R., Chong, J. A., and Mandel, G. (1994). Neural expression of a sodium channel gene requires cell-specific interactions. *Neuron* **13,** 937–948.

Ortolani, G., Patricolo, E., and Mansueto, C. (1979). Trypsin-induced cell surface changes in ascidian embryonic cells: Regulation of differentiation of a tissue-specific protein. *Exp. Cell Res.* **122,** 137–147.

Pandur, P., and Moody, S. (2000). Xenopus Six1 gene is expressed in neurogenic cranial placodes and maintained in the differentiating lateral lines. *Mech. Dev.* **96,** 253–257.

Pera, E. M., Wessely, O., Li, S. Y., and De Robertis, E. M. (2001). Neural and head induction by insulin-like growth factor signals. *Dev. Cell* **1,** 655–665.

Pourquie, O. (2001). Developmental biology. A macho way to make muscles. *Nature* **409,** 679–680.

Reverberi, G., and Minganti, A. (1946). Fenomeni di evocazione nello sviluppo dell'uovo di ascidie. Riulti dell'indagine sperimentale sull'uovo di *Ascidiella aspersa* e do *Ascidia malaca* allo stadio di otto blastomeri. *Pubbl. Stn. Zool. Napoli* **20,** 199–252.

Reverberi, G., and Minganti, A. (1947). La distribuzione delle potenze nel germe di Ascidie allo stadio di otto blastomeri, analizzata mediante le combinazioni e i trapianti di blastomeri. *Pubbl. Stn. Zool. Napoli* **21,** 1–35.

Reverberi, G., Ortolani, G., and Farinella-Feruzza, N. (1960). The causal formation of the brain in the ascidian larva. *Acta Embryol. Morphol. Exp.* **3,** 296–336.

Rose, S. M. (1939). Embryonic induction in the ascidia. *Biol. Bull.* **76,** 216–232.

Sasai, Y. (1998). Identifying the missing links: Genes that connect neural induction and primary neurogenesis in vertebrate embryos. *Neuron* **21,** 455–458.

Sato, S., Masuya, H., Numakunai, T., Satoh, N., Ikeo, K., Gojobori, T., Tamura, K., Ide, H., Takeuchi, T., and Yamamoto, H. (1997). Ascidian tyrosinase gene: Its unique structure and expression in the developing brain. *Dev. Dyn.* **208,** 363–374.

Sato, S., Toyoda, R., Katsuyama, Y., Saiga, H., Numakunai, T., Ikeo, K., Gojobori, T., Yajima, I., and Yamamoto, H. (1999). Structure and developmental expression of the ascidian TRP gene: Insights into the evolution of pigment cell-specific gene expression. *Dev. Dyn.* **215,** 225–237.

Sato, S., and Yamamoto, H. (2001). Development of pigment cells in the brain of ascidian tadpole larvae: Insights into the origins of vertebrate pigment cells. *Pigment Cell Res.* **14,** 428–436.

Satou, Y., Imai, K. S., and Satoh, N. (2001a). Action of morpholinos in Ciona embryos. *Genesis* **30,** 103–106.

Satou, Y., Imai, K. S., and Satoh, N. (2001b). Early embryonic expression of a LIM-homeobox gene Cs-lhx3 is downstream of beta-catenin and responsible for the endoderm differentiation in *Ciona savignyi* embryos. *Development* **128,** 3559–3570.

Satou, Y., Takatori, N., Yamada, L., Mochizuki, Y., Hamaguchi, M., Ishikawa, H., Chiba, S., Imai, K., Kano, S., Murakami, S. D., Nakayama, A., Nishino, A., Sasakura, Y., Satoh, G., Shimotori, T., Shin, I. T., Shoguchi, E., Suzuki, M. M., Takada, N., Utsumi, N., Yoshida, N., Saiga, H., Kohara, Y., and Satoh, N. (2001c). Gene expression profiles in *Ciona intestinalis* tailbud embryos. *Development* **128,** 2893–2904.

Satou, Y., Imai, K. S., and Satoh, N. (2002). Fgf genes in the basal chordate *Ciona intestinalis*. *Dev. Genes Evol.*, **212,** 432–438.

Schier, A. F., and Talbot, W. S. (2001). Nodal signaling and the zebrafish organizer. *Int. J. Dev. Biol.* **45,** 289–297.

Schneider-Maunoury, S., Gilardi-Hebenstreit, P., and Charnay, P. (1998). How to build a vertebrate hindbrain. Lessons from genetics. *C. R. Acad. Sci. III* **321,** 819–834.

Selleck, M. A., and Stern, C. D. (1991). Fate mapping and cell lineage analysis of Hensen's node in the chick embryo. *Development* **112,** 615–626.

Shimauchi, Y., Murakami, S. D., and Satoh, N. (2001). FGF signals are involved in the differentiation of notochord cells and mesenchyme cells of the ascidian *Halocynthia roretzi*. *Development* **128,** 2711–2721.

Shimauchi, Y., Yasuo, H., and Satoh, N. (1997). Autonomy of ascidian fork head/HNF-3 gene expression. *Mech. Dev.* **69,** 143–154.

Shu, D. G., Chen, L., Han, J., and Zhang, X. L. (2001). An early Cambrian tunicate from China. *Nature* **411,** 472–473.

Simmen, M. W., Leitgeb, S., Clark, V. H., Jones, S. J., and Bird, A. (1998). Gene number in an invertebrate chordate, *Ciona intestinalis*. *Proc. Natl. Acad. Sci. USA* **95,** 4437–4440.

Sive, H. L., Hattori, K., and Weintraub, H. (1989). Progressive determination during formation of the anteroposterior axis in *Xenopus laevis*. *Cell* **58,** 171–180.

Sordino, P., Belluzzi, L., De Santis, R., and Smith, W. C. (2001). Developmental genetics in primitive chordates. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **356,** 1573–1582.

Takamura, K. (1998). Nervous network in larvae of the ascidian *Ciona intestinalis*. *Dev. Genes Evol.* **208,** 1–8.

Torrence, S. A., and Cloney, R. A. (1982). Nervous system of ascidian larvae: Caudal primary sensory neurons. *Zoomorphology* **99,** 103–115.

Wada, H., Holland, P. W., Sato, S., Yamamoto, H., and Satoh, N. (1997). Neural tube is partially dorsalized by overexpression of HrPax-37: The ascidian homologue of Pax-3 and Pax-7. *Dev. Biol.* **187,** 240–252.

Wada, H., Saiga, H., Satoh, N., and Holland, P. W. (1998). Tripartite organization of the ancestral chordate brain and the antiquity of placodes: Insights from ascidian Pax-2/5/8, Hox and Otx genes. *Development* **125,** 1113–1122.

Wada, H., and Satoh, N. (2001). Patterning the protochordate neural tube. *Curr. Opin. Neurobiol.* **11,** 16–21.

Wada, S., Katsuyama, Y., and Saiga, H. (1999). Anteroposterior patterning of the epidermis by inductive influences from the vegetal hemisphere cells in the ascidian embryo. *Development* **126,** 4955–4963.

Wada, S., Katsuyama, Y., Sato, Y., Itoh, C., and Saiga, H. (1996). Hroth an orthodenticle-related homeobox gene of the ascidian, *Halocynthia roretzi*: Its expression and putative roles in the axis formation during embryogenesis. *Mech. Dev.* **60,** 59–71.

Wada, S., and Saiga, H. (1999a). Cloning and embryonic expression of Hrsna, a snail family gene of the ascidian *Halocynthia roretzi*: Implication in the origins of mechanisms for mesoderm specification and body axis formation in chordates. *Dev. Growth Differ.* **41,** 9–18.

Wada, S., and Saiga, H. (1999b). Vegetal cell fate specification and anterior neuroectoderm formation by Hroth, the ascidian homologue of orthodenticle/otx. *Mech. Dev.* **82,** 67–77.

Whittaker, J. R. (1987). Neural induction in ascidian embryos redivivus. *Biol. Bull.* **173,** 428–429.

Whittaker, J. R., Ortolani, G., and Farinella-Ferruzza, N. (1977). Autonomy of acetylcholinesterase differentiation in muscle lineage cells of ascidian embryos. *Dev. Biol.* **55,** 196–200.

Wilson, S. I., and Edlund, T. (2001). Neural induction: Toward a unifying mechanism. *Nat. Neurosci.* **4**(Suppl.), 1161–1168.

Yagi, K., and Makabe, K. W. (2001). Isolation of an early neural maker gene abundantly expressed in the nervous system of the ascidian, *Halocynthia roretzi*. *Dev. Genes Evol.* **211,** 49–53.

Received for publication September 12, 2002
Accepted September 21, 2002
Published online November 14, 2002